

What happens to NASA now?

This week's report on the shuttle disaster is diagnostic rather than prescriptive. The most urgent need is that NASA should be broken into more manageable pieces.

THE Rogers Commission has at least been swift: it was told to be. While the shuttle catastrophe is still fresh in the mind, there has appeared an intelligent diagnosis of the trouble and a recipe for its avoidance in future. Psychologically, this is what the people of the United States have been yearning for ever since Challenger, with its crew of seven, was destroyed off the coast of Florida in January. The catastrophe was a blow to national self-esteem which has since been made to seem the more cruel by the capture of US citizens as hostages in the Middle East and other assorted insults. Much as it always helps to know the truth, the realism of the Rogers report will persuade people that even if the problems uncovered are serious, they are not insuperable. That is a plus by anybody's reckoning.

The downside is the severity of the criticism directed at the US National Aeronautics and Space Administration (NASA), even if Dr Richard Feynman, a member of the commission, would have preferred a more outspoken report. For several months, it has been clear that the commission would point to errors of two kinds, technical and managerial. The tale of how the different sections of the booster rockets were held together by mechanical joints including rubber seals to prevent the escape of combustion gases has been widely discussed, not least because of the commission's public hearings; these rockets were poorly conceived and their behaviour in exceptional circumstances poorly understood. The managerial errors, for which several senior NASA people have already lost their jobs (the director of the Marshall Space Flight Center in Alabama did well to quit before the Rogers report appeared), have also been apparent for some time. There are many other large organizations in which bad news travels only slowly upwards in the hierarchy, but by the beginning of this year, NASA appears to have been persuaded by the pressure to keep on launching shuttles to devise mechanisms for preserving the innocence among its senior people of the realities of the launching-pad. It is no wonder that the Rogers report is scathing on the subject.

There is every likelihood that the steps recommended by the Rogers Commission will right these faults. The boosters will be redesigned, while there is almost certain for a few months to be a mood in which doubts and criticisms will move freely within the organization and be openly discussed. Given past achievements, there is no reason why NASA should not be able to put these things to rights, so that shuttles will be climbing safely through the atmosphere into Earth orbits a year or so from now. But these are relatively minor and even technical matters, well within NASA's competence. What nobody has considered yet, but what the US administration should take up urgently, is the more teasing question of what NASA is for, and whether its organization is compatible with those goals.

Goals

It tends to be forgotten, but should not be, that NASA as it now is grew out of a federally-supported aeronautical research organization in the United States, and that basic research in the field remains an important part of its function. Much the same is true with rocketry and spacecraft construction. Of necessity, NASA has had to pave the way with these developments. One of

its perennial difficulties is that it has been able to shed responsibility for only a small part of its role in technological research and development, mostly the work on satellite design and construction on which the telecommunications companies are engaged. But NASA has also, almost without recognizing it, become an operating agency, continuing to operate satellites that cannot be handed over to commercial interests (which means most of them), launching and recovering spacecraft and so on. That, through the agency of a talented if geographically scattered staff, NASA has also become an essential complement to the academic community, is an uncovenanted benefit.

Defects

NASA's defects, unfortunately, are also many and familiar. Whether it is fair to say, as many newspapers have been doing, that NASA has become arrogant and over-confident is irrelevant. With 15 years of almost unalloyed success in launching well-publicized spacecraft to its credit, it must be tempting to be cocky. But there is plenty of evidence that goes the other way. It is a long time since NASA as an organization behaved as if it were possible to fight the US administration and the Congress for a budget that matched the ambitions with which it had been saddled by public policy. Of necessity always a big spender, NASA has nevertheless become almost a passive spender, willing to cut its coat to suit the cloth the appropriations committees handed out even when this meant abandoning important projects, most often important scientific projects, sometimes projects to which agencies elsewhere (such as the European Space Agency) had been induced to commit funds. Is it possible that NASA's learned behaviour of pleading poorness and blaming the US Congress for that condition led it also to the view that trouble caused by cutting corners, as in January, could also be blamed on others? The Rogers Commission says the pressure was to run shuttle launches as if by an airline timetable so as to cultivate congressional indulgence. It is a shame that, because this calculation has misled, parts of NASA's operations that are well conceived and imaginatively run may be tainted by the same charge of mismanagement.

The truth is that NASA has acquired too many partially conflicting goals. Space launchers are at best means to a variety of ends, but when the launcher programmes are in trouble, the ends tend to be sacrificed to the means. The solution is to break up the present organization into a few smaller pieces whose objectives would be more coherent. One way of doing this would be to separate technical development (including that of aircraft), the business of running transportation systems and NASA's role as a doer and sponsor of space research (which at present is hurt first when funds are short). Then at least there would be only the budget-people to blame if the parts of the programme swung out of balance. NASA will no doubt protest that the same argument could be used to take high-energy physics away from the Department of Energy, but that is not the case; the department, like its predecessor, the Atomic Energy Commission, has sensibly devised a mechanism by which the high-energy physics community's claims on public funds stand or fall by the good sense of what the High-Energy Physics Advisory



Panel thinks it proper to recommend from time to time.

NASA may also fear that dismemberment into more manageable pieces may complicate its matching of the demand for launching facilities with the supply it has created. That, too, is misplaced. While the vast majority of the projects on which researchers have set their sights could be handled as well by inanimate rockets as by the shuttle, that is not the case for the most valuable of all the projects, the Hubble Space Telescope (which must now languish on the ground for an extra year), while there would be no case against a craft such as the shuttle if it were dependable and cheap. In any case, especially after the latest failure of Ariane two weeks ago, launcher operators are unlikely to have spare capacity for a long time to come.

This is why the best framework in which to solve NASA's problems is quick clean surgery, which will in any case be necessary at some stage in the future, when spacecraft with or without people have ceased to be seven-day wonders. □

Observers in arms

The fate of Britain's oldest observatory will be decided next week. Or will it?

How strong are the nerves of the Science and Engineering Research Council (SERC), the chief sponsor and even manager of Britain's academic research? This is what British astronomers will be asking in the time between now and 18 June, the date fixed for the next meeting of the council and, at SERC's own choice, for a final decision on the future of the Royal Greenwich Observatory (RGO). In the three months since the decision was announced (see *Nature* 320, 239; 1986) that the observatory would have to move again — forty years have gone since it said goodbye to Greenwich — there has been a fierce rumbling of protest from many of those affected that the decision is mistaken, which message has been blunted and confused by the siren-sounds from those other astronomers with an interest in tempting RGO to some particular place or other. A meeting of astronomers of all kinds in London last Friday seemed to be united in feeling that SERC has somehow mismanaged the whole business. So next week's council meeting becomes a test of SERC's credibility as well as of its good sense.

The reasons why some kind of decision is needed are by now well-known. Because of decisions taken by SERC (then merely SRC) in the 1970s, British astronomers are about to be blessed with a range of what may reasonably be called modern telescopes, and which will also give academic astronomers allocations of telescope time about which they cannot seriously complain. The snag is that the new equipment is a long way from Britain, in the middle either of the Atlantic or of the Pacific. As observers elsewhere know as individuals, geography may be a nuisance but is not an insuperable obstacle to good work. SERC's difficulty is that, having built the new telescopes as part of its terms of reference to foster research at British universities and polytechnics, it discovers on its payroll some 250 people at the two British observatories, RGO and the Royal Observatory Edinburgh. The problem might have been anticipated a decade ago, but was not. Since then, by common consent, the two observatories have done valuable work in supporting the development of instruments for the facilities being built elsewhere and, more recently, have been providing academic users of the new facilities with advice and assistance based on their own experience, frequently much greater. That there will be a continuing need for services of this kind is generally accepted. SERC's problem is three-fold. First, as a consequence of past decisions, it is spending more on astronomy relative to other things than it would if it were starting from scratch (but nothing said about the decision to move RGO suggests immediate savings). Second, without the issue ever having been declared, SERC is seeking (rightly) to effect a shift of responsibility (or opportunity) for observational astronomy from the in-house

observatories to the universities, which is why the decision that Greenwich rather than Edinburgh should move was based on SERC's opinion (which RGO disputes) that Greenwich has been the less successful at forming links with universities. Third, at no point during the five years in which the need for reorganization has been apparent, during which time there have been two formal attempts to find an acceptable solution, has there been a serious effort to decide how the now-expanded British effort in astronomy should be managed. There is a sense in which the present council is the victim of its predecessors' neglect which, given collective responsibility, is an explanation but not an excuse. The anger and bitterness that have preceded next week's council meeting have arisen because too little has been said to those whose careers are affected.

What, then, should be done to manage the transition without further exacerbating relations between SERC and important sections of the astronomy community? The first need is for a convincing statement of SERC's strategy for astronomy. To what extent is the long-term aim, that most observers should be academics, desirable, for example? Having made a good if belated beginning on the provision of modern facilities, does SERC intend to follow through when the demands come (as they surely will) for even better equipment? And may it then not act alone, picking up assistance from others as an afterthought (Dutch as well as Spanish astronomers are partners in the La Palma observatory), but by seeking partners from the outset? All three questions should of course be answered yes. But only if they are answered clearly and sympathetically will those at the observatories whose careers will be affected have the stomach to soldier on.

What would such a strategy imply for the immediate future? If the best way of building on past investments in astronomy without jeopardizing science as a whole is to build the next generation of telescopes in collaboration with partners overseas, SERC would recognize more clearly that national support centres are inappropriate. Planning that observers should be distributed among universities, it would conclude that technical support should be similarly spread out. In the long run, neither Edinburgh nor RGO would be needed. The objection to SERC's planned course, amalgamation on the Edinburgh site, is that it would permanently institutionalize the complex. If it were now to say that Edinburgh will in due course follow RGO in being made part of a university, RGO's bitter pill would be easier to swallow. If SERC is not ready to say that, the case for moving RGO diminishes.

SERC should also be more open about its financial plans. It says that present recurrent spending will not increase, but no allowance has yet been made for future capital expenditure. Why not say flatly that SERC needs to save money now on running costs against capital spending in the future? RGO itself will have shrunk to 150 people (of whom 40 will be based in mid-Atlantic) when the present contraction is complete by 1990, but there is a case for going faster and further. The millstone that SERC has made to hang around its successors' necks is the promise that RGO will retain its independence, which will be taken as a promise of constant budgets a decade hence.

There remains the question of how British astronomy should acquire a sense of being led in a recognizable direction. SERC is a benign and well-intentioned organization that has done good works from time to time. Unfortunately, it cannot help but seem part of the civil service: why else have the directors of the two observatories been compelled to say nothing in the past three months about the aspect of their institutions that matters most, their future? Like NASA, which has the more serious problem of the shuttle to accommodate (see previous page), SERC needs a mechanism for helping the community it tries to serve to believe that its true wishes, not some others wished upon it, will be met. If SERC could arrange for that, its difficulties with those at RGO would melt away. If it cannot, its credibility will indeed decline. □

Shuttle catastrophe

Rogers Commission report makes waves all round

Washington

DESIGN flaws, organizational problems and inadequate safety procedures all played a role in the accident on 28 January that destroyed the space shuttle Challenger, according to the presidential commission chaired by William P. Rogers, whose 250-page report was published this week.

The commission concludes that the accident to mission 51L occurred when a gas leak developed at the joint connecting two segments of the right solid rocket booster (SRB). The leak developed within seconds of launch, showing up in photographs as a puff of black smoke. Then it evidently sealed itself, possibly with combustion products from the booster, but after the shuttle successfully passed through severe wind shear, the leak reappeared 58 seconds after lift-off.

Then the slipstream directed the burning gas from the leak onto the external fuel tank, ultimately burning a hole in it. At 72 seconds, the lower strut holding the booster to the fuel tank was severed or pulled away, allowing the booster to pivot, smashing into the external fuel tank and releasing massive amounts of liquid hydrogen. Within milliseconds there was a "massive, almost explosive burning" of the hydrogen spilling from the tank.

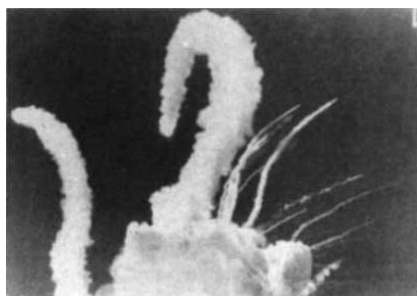
The commission concluded that the accident occurred because O-rings supposed to form a seal between two segments of the right solid rocket booster failed to do so. The O-ring seals are designed to be "pressure actuated", only sealing once increased pressure is applied to the joint by the firing of the rocket motor. The commission concluded that the failure was due to "a faulty design".

The commission was also critical of the process that resulted in the decision to launch Challenger. Testimony presented to the commission revealed that the decision to fly was based on "incomplete, and sometimes misleading information". The O-rings had been identified as early as December 1982 as parts whose failure could result in a mission disaster. In July 1985, SRB manager Lawrence Mulloy placed a launch constraint on all shuttle launches, which meant that a waiver had to be signed before each flight could proceed. But neither the constraint nor the waivers were known to the director of the shuttle programme or his senior deputies.

The 24 previous successful launches of the shuttle also contributed to the accident. As commission member Richard Feynman put it, NASA convinced itself

that safety standards could be lowered a little bit after each launch "because we got away with it last time".

The commission also investigated the pressures on the shuttle programme caused by NASA's reluctance to relax optimistic schedules. A critical shortage in spare parts forced NASA to turn to cannibalization, borrowing parts from one



orbiter to allow another one to fly. But the accelerated launch schedule meant the shuttle would either be in orbit or preparing for launch, thus not available for supplying spare parts.

The commission's chief recommendation is that the faulty joint and seal of the solid rocket booster should be either eliminated or redesigned. But other recommendations are likely further to increase the cost of bringing the shuttle back into service and to decrease the rate of shuttle launches thereafter.

The booster joints had for several years been suspect. Instead of a complete redesign of the joint, now as seen as necessary, Morton Thiokol Inc. and NASA opted for some retesting and the imposition of "launch constraints", which were then overridden for every launch after 10 July 1985.

The commission is forthright on the need for other safety improvements, landing safety included. Tyres on the landing gear are acknowledged by NASA to be "critical" components, but tyres have shown "excessive" wear during several previous landings. The brakes, which have been damaged on most flights, have "little or no margin".

The commission urges efforts to provide a crew escape system for use during gliding flight. Although early test flights of the shuttle included ejection seats, recent space missions have had no such feature, which would require a major effort, and probably taking up valuable space and weight. "Rocket-assisted extraction" is a possibility.

The commission, impressed that pressure on NASA to maximize launch rates

may have contributed to management failures and the accident, says the capabilities of the system stretched "to the limit" to support flight rate in winter 1985-86. In what may be one of the most significant long-term recommendations, the commission urges that the United States should not rely exclusively on the shuttle for launching capacity, a complete reversal of the administration's declared policy.

On management, Rogers says there must be a redefinition of the programme manager's responsibility. Project managers apparently felt more accountable to their particular NASA centre than to the programme as a whole.

Tim Beardsley & Joseph Palca

Other avenues?

Washington

THE Rogers inquiry subdued the natural inclination of Congress to get involved; now that the report is public, both houses of Congress intend to hold hearings on the findings, and on the accident itself.

At least one senator, unable to wait until publication day on Monday, introduced legislation last week following a "recommendation by the Rogers Commission" to revive quality assurance at NASA. Congress has also been impatient with the inability of the White House to decide on buying a new orbiter, and may suggest its own plans for the agency.

The National Research Council (NRC) will also have a future role in the shuttle programme. NRC's standing review committee on NASA's scientific and technological programme will set up a panel to evaluate development and construction of the new solid rocket boosters.

NASA itself is also having to reevaluate its role. The Reagan administration no longer wants NASA to be the sole provider of launch services for commercial satellites, while the Department of Defense has already ordered ten Titan 34D7 rockets capable of launching payloads nearly as large as those carried by the shuttle.

Without the military or industry as steady users, will NASA find itself more interested in the needs of the scientific community? Bruce Murray, professor of planetary science at the California Institute of Technology and former director of the Jet Propulsion Laboratory, says NASA took a procrustean approach, forcing users to fit their projects to the shuttle's capabilities. Murray believes NASA will now have to serve space science and manned exploration of space.

For the time being, with no shuttle and no alternative launch vehicles, space science projects are dead in their tracks. Even if funds for scientific projects are preserved, NASA's chief scientist Frank McDonald says the shuttle's loss will damage the programme.

Joseph Palca

Seismic monitoring

Private diplomacy emergent

Washington

A REMARKABLE agreement signed last month would permit a team of US and Soviet seismologists to establish a seismic monitoring station within 200 km of the Soviet nuclear test facility at Semipalatinsk. What makes the agreement all the more unusual, and contentious, is that it was not signed by representatives of the US government.

The agreement came out of a series of meetings between representatives of the Natural Resources and Defense Council (NRDC), a private non-profit organization devoted to protecting the environment, and the Soviet Academy of Sciences. Adrian DeWind, NRDC's board chairman, and E.P. Velikhov, vice-president of the Soviet Academy, signed the agreement in Moscow on 28 May.

Under the agreement, six monitoring stations would be established, three in the Soviet Union near Semipalatinsk and three near the Nevada test site in the United States. All data recorded will be made available to both the US and Soviet governments. NRDC will pay all the travel expenses involved in the project and will take responsibility for obtaining the necessary seismological equipment. DeWind estimates that NRDC will have to raise \$500,000 to get the project off the ground. It hopes to establish the first listening post in the Soviet Union by the end of this month, with two more to follow in the fall. The US monitoring posts will be established only when the Soviet posts are in place.

Thomas Cochran, a physicist on NRDC's senior staff, says the idea for the agreement came from discussion last January on the monitoring of US explosions whose occurrence is not made public. Cochran and his colleagues at NRDC felt that monitoring only the Nevada test site might be regarded as "giving away state secrets", so he devised the bilateral plan.

Although the monitoring sites may provide information about the local geology of the Semipalatinsk region useful for evaluating yields of Soviet nuclear tests, that is not the point of the exercise. Rather, says Cochran, it is to show the feasibility of on-site seismic monitoring, proving that verification is not an obstacle to a test-ban treaty. The Soviet Union is currently observing a self-imposed moratorium on tests, but that may expire on 6 August.

Before the plan can go forward, NRDC must obtain export licences for the seismic equipment. A State Department official said last week that no decision had been made on NRDC's request for a licence, but that the agencies responsible for tech-

nology transfer issues were considering it. NRDC plans to purchase off-the-shelf equipment for the monitoring stations, according to Charles Archambeau of the University of Colorado who acted as a consultant to NRDC. If the export licences are not granted, Archambeau says the project could still proceed using Soviet-built equipment. But Archambeau says that Soviet equipment is not as sensitive as that from the United States, nor is it digital, making data acquisition and analysis more difficult.

Critics of the agreement believe that it will serve only as a propaganda weapon for the Soviet Union. Misha Tsygkin, Salvatori Fellow in Soviet Studies at the Heritage Foundation, a conservative think-tank, argues that the Soviets are adept at manipulating foreigners to serve Soviet goals. Tsygkin believes that NRDC was "irresponsible and out of order" to proceed with such politically sensitive negotiations off its own bat, without government participation or approval. Archambeau concedes that NRDC's plans may undercut current US government positions on arms control. But according to Cochran, something was needed to "get some movement in the arms control arena".

The agreement comes at a time when the United States has announced it plans to end its observance of the unratified SALT II treaty later this year. The government believes that Soviet unwillingness to abide by the terms of the treaty makes it impossible for the United States to do so. But efforts are under way in Congress to force US compliance with the treaty by eliminating funds for projects that would violate its terms; these are unlikely to succeed, however. Supporters of the Soviet test moratorium are also planning to introduce legislation stopping US nuclear testing, although these efforts also face an uphill struggle.

According to a State Department official, the NRDC agreement "may be encouraging" to the extent that it represents a change in Soviet attitudes on verification of the Threshold Test Ban Treaty, which the United States believes the Soviet Union has been violating. But the State Department believes that negotiations that could have such a great impact on arms control and national security should be conducted only between governments.

Frank Press, president of the National Academy of Sciences, sees the NRDC agreement as an "extraordinary event", but says it is too soon to judge its implications. "Arms control has never been a private sector activity before... When books on arms control are written, this will certainly be included." **Joseph Palca**

Soviet industry

Reformers get the wrong man

IT seems that Mr Mikhail Gorbachev's drive to make Soviet industry more efficient is not without its hitches. Last month, none other than the Central Committee of the Communist Party of the Soviet Union intervened to demand the reinstatement of a person who had earlier been wrongfully demoted from his managerial post at the Irbit Chemical and Pharmaceutical Plant.

The Central Committee's special decree dealing with the state of affairs at the plant is revealing. The plant is supposed to produce high-quality supplies for the Soviet medical services, but is run-down, behind in its modernization programme and provides no scope for the development of new ideas and products. These are precisely the conditions condemned by the plenum of the Central Committee held in April 1985.

The Central Committee now says that, instead of trying to rectify these conditions, the director of the plant, with the support of the local party organization, deprived a certain Vadim Bakharev of his post, in charge of production unit Number 2, on the grounds that "he had ignored the opinion of working teams", shown "crudeness" in dealings with his subordinates and had "consistently failed to fulfil his work obligations". Bakharev accepted his transfer to the lower grade of engineer only because of the threat that he would otherwise be dismissed outright.

Bakharev was demoted on 4 December last year. How and when the higher echelons of the party learned what was going on is not clear, but, on 20 April, the newspaper *Pravda* carried a long account of the affair which claimed that, far from being responsible for the shortcomings at the Irbit plant, Bakharev was a lone hero who had fought neglect and inefficiency, and who had been victimized for his zeal.

According to *Pravda*, Bakharev had in reality combined two previous production units together so as to cut costs by 75 per cent and increased labour productivity by 98 per cent (partly by eliminating 36 jobs) at a saving of 300,000 rubles a year. It was not Bakharev but the plant managers who had "ignored the opinion of the working teams" by demoting such a paragon.

A month later, the Central Committee pronounced. The Ministry of Medical and Microbiological Industry was instructed that Bakharev's demotion had been unjustified, and that he must be reinstated forthwith. The ministry was also told to put matters at the factory right, modernizing and re-equipping the production lines and improving the working and living conditions of the workforce. The party

secretary at the plant has been relieved of his post and other officials disciplined.

The Irbit factory is important in the Soviet Union's plans to build a sophisticated pharmaceutical and specialist chemical industry, and should be at the leading edge of the government's new drive for modernization. The intervention of the Central Committee, and the attention it has been given, may be intended as a warning to plant managers elsewhere that they had better put their affairs in order before the drive for modernization gathers momentum and that a random selection of scapegoats will not suffice.

Vera Rich

AIDS research

New foundation begins work

Washington

SUPPORT for research into acquired immune deficiency syndrome (AIDS) has received a shot in the arm from the American Foundation for AIDS Research (AmFAR). The new foundation handed out 20 grants totalling \$1.1 million, mostly for basic research on the biology of the virus associated with AIDS, with a smaller number for drug development and epidemiology. AmFAR's scientific advisory committee was looking primarily for projects demonstrating "fresh ideas", according to a spokesman for AmFAR.

The 20 projects receiving grants were chosen from 146 applications. The maximum \$60,000 grant is not intended to sustain a major research effort, but will instead provide start-up money for new projects.

AmFAR came into existence last September when the AIDS Medical Foundation, based in New York, merged with the National AIDS Research Foundation of Los Angeles. Actress Elizabeth Taylor serves as AmFAR's national chairman, and has been an active force in fund-raising. In future, AmFAR hopes to move its financial base away from special fund-raising events to long-term commitments from corporations and philanthropic foundations.

Although the \$1.1 million offered by AmFAR is only a small fraction of the \$233 million AIDS budget of the US Public Health Service, there is always enthusiasm for a new source of funds. "Any branch I can shake for support is marvelous," says Murray Gardner of the University of California at Davis, who received a \$60,000 grant from AmFAR to study the structure of the envelope protein of the AIDS virus.

AmFAR can also move more quickly than the federal government. It solicited proposals in January, and made decisions on them just 4 months later. **Joseph Palca**

Rice breeding

Manipulation of cereal succeeds

Tokyo

THE basic techniques for the genetic engineering of rice have been developed in research institutes in Japan. Several laboratories are racing to draw the techniques together so as to be the first to produce a genetically engineered rice plant. The development is important not only because genetic manipulation has so far been mostly confined to dicotyledonous plants but also because rice is the staple food of half the world's population.

The transfer of genetic material into cereal plants has hitherto been held up because they cannot be infected with the *Agrobacterium* species which have provided a simple route to the transfer of genes in dicotyledonous plants. But two new developments have given rise to optimism that genetic manipulation of rice will be possible in the near future.

The regeneration of whole rice plants from protoplasts (single rice cells stripped of their cell wall) is one way. Success has just been reported in print by Professor Yasuyuki Yamada's group at the Kyoto University Research Center for Cell and Tissue Culture (see *Plant Cell Reports* 5, 85; 1986). Two industrial laboratories, a joint laboratory run by Mitsubishi Chemical and Mitsubishi Corporation and Mitsui Toatsu's research laboratory have also described regeneration techniques at research meetings. Each group has its own method and it is too early to know which will prove most practical.

Yamada can regenerate tissue from protoplasts by the simple trick of adding calf serum, commonly used in the culture of animal cells, to his medium; after all, he says, a "plant protoplast is almost the same as an animal cell".

From one culture cell line, Yamada has been able to produce calli (undifferentiated tissue) from 10 per cent of protoplasts, many of which formed roots and shoots and developed into whole plants when transferred to regeneration medium. Although other strains produced calli, only one regenerated whole plants and there is evidence that the adult plants, now out in pots and the fields, are not entirely normal. Further work may thus be needed to make the regeneration method of general use.

The Mitsubishi group, led by Dr K. Shimamoto, appears to have had much broader success. It has successfully regenerated whole plants from protoplasts of five different genotypes, two of them major commercial cultivars. More than 300 plants have now been regenerated, and of these 120 are now growing in paddy fields. They can later be studied in detail for differences from the parent stock.

The innovations behind the Mitsubishi

group's success is that, instead of making protoplasts from rice cell lines maintained for a long time in culture, they have induced calli from rice seeds. The protoplasts so produced are "young" and have strong morphogenic potential. In the past, this approach has not been favoured because cells in fresh culture from seed do not divide — overcoming this obstacle is the secret of Shimamoto's method.

The second group of advances, for the transfer of foreign genes into protoplasts, has also very recently been reported, and depends on the observation that protoplasts prepared from cultured callus cells and treated with polyethylene glycol will take up foreign DNA. Dr Uchimiya's group at the University of Tsukuba has successfully introduced a chimaeric gene containing an antibiotic resistance gene in experiments of this kind. He was not, however, able to regenerate plants from the transformed cells.

With both regeneration and transformation proved possible, the race is now on to "put both techniques in one hand", as Uchimiya says, and to produce the first genetically engineered rice plants. Judging from reports from the five or so laboratories carrying out similar work, the winner should be declared fairly soon.

The question remains of what useful application can come from the research. In theory, the potential is vast, for rice is the major source of carbohydrate for almost half of the world's population. But rice has been intensively improved by breeders for almost a century, while large increases in production can be expected in the poorer countries of Asia simply through extending irrigation. This makes Japanese companies wary about investing heavily in rice research, for even now rice can be grown in Japan only by maintaining an artificial price several times that of the world market.

Early on, genetic engineering may be used to make things easier for the conventional breeder. Male sterile lines, valuable in breeding new strains of rice, are hard to produce by ordinary methods but should be easily made by protoplast fusion. One protoplast carrying the cytoplasmic male sterility factor but with its nucleus inactivated can be fused with the desired strain of rice.

In the longer term there are the possibilities of transferring genes from other cereal crops that seem to perform more efficiently, introducing viral resistance genes and altering genes to improve the quality of the seed protein. Again, there are ten wild species of rice which present an almost untapped reserve of genetic variability. Protoplast fusion will make it accessible. **Alun Anderson**

Germany and Chernobyl

End of the nuclear programme?

Hamburg

THE cloud whose changing direction carried radioactivity from Chernobyl over Western Europe last month has changed the political climate in West Germany. Fallout has become a national issue which will affect the regional (*länder*) elections due in the next few months and even the federal elections next January.

The general confusion in the days following the accident has left its mark. Chancellor Helmut Kohl, in Tokyo for the economic summit, at first reacted calmly, saying that the accident was characteristic of planned economies and could not have happened in West Germany. By the time, six days after the accident, that fallout reached West Germany, Kohl (still in Tokyo) was urging further expansion of nuclear energy in West Germany even as the first warnings against drinking fresh milk were being published.

Conflicting information was given out from several sources. The Minister of the Interior, Friedrich Zimmermann (CSU), was explaining on television that contamination from a reactor 2,000 miles away could not occur while the first radioactive rain began to fall. Officials of the federal and *länder* governments gave out contra-



Police clash with demonstrators at Brokdorf last week

dictory advice. In Bavaria, the most affected of the *länder*, the right-wing government played down the problem for the first few days, but in Munich, with its SPD (Social Democrat) city government, people were told to avoid contact with soil or grass.

The federal government said that people should not drink milk whose radioactivity was more than 500 Bq per litre, but in some *länder* the limit was only half as much, while Hesse fixed it at 20 Bq per litre. In many cities, kindergartens and playing-fields were closed and the marketing of green salad and spinach forbidden. Farmers were forced by the police to destroy vegetables or, as in Nordrhein-Westfalen, to keep cattle off pasture. Geiger counters were sold out after a few days.

Psychoanalysts claim to have identified a novel fear among the people of West Germany, whose dread has been described by the newspaper *Bild* as "Atom-Angst" (nuclear fear).

The political consequences are signalled by the latest opinion polls, to which the political parties have begun to adapt their nuclear policies. The Greens, who have always wanted an end to nuclear construction, now demand that nuclear plants should be abandoned. The Social Democrats (SPD), conscious of the figures, have adopted the slogan "the beginning of the end" and have appointed a committee under Volker Hauff, a former federal minister of research, to suggest by the summer how the nuclear programme should be run down.

Public opinion has changed dramatically. The percentage of those wanting no further nuclear reactors to be built grew in a few days from 46 per cent to 69 per cent and reached a peak of 83 per cent. In Niedersachsen (Lower Saxony), where there is an election on 15 June, the opinion polls put CDU (Christian Democrats) and SPD almost equal with 43 per cent of popular support, compared with 51 per cent for CDU and only 37 per cent for SPD at the 1982 elections. The same poll gives FDP (Free Democrats) less than the 5 per cent required for representation in the regional parliament. The Greens have increased their support to 10 per cent from 6.5 per cent in 1982.

This week's elections in Niedersachsen could have national consequences. If SDP in coalition with the Greens (as in Hesse) is able to oust the present CDU chief minister, the government coalition could lose its majority in the Bundesrat in Bonn.

SDP is looking further ahead, to the federal elections in January 1987. It has already said that it would probably stop the DM7,000 million fast reactor project at Kalkar, but Johannes Rau, the SDP candidate for the chancellorship next year who is at present chief minister of Nordrhein-Westfalen, where Kalkar is situated, has already ensured that the builders of the project will be forced to give it up by demanding new safety measures.

With the Kalkar reactor apparently doomed, attention has turned to the reprocessing plant at Wackersdorf in Bavaria, the technical need for which turns on the manufacture of plutonium for the fast reactor. There have been violent clashes at the plant between police and demonstrators. The Austrian government has also demanded that West Germany should give up the project, but has been told not to interfere. Both Bonn and the CSU Bavarian government seem committed to the DM6,000 million project, al-

though that may change before the autumn's election in Bavaria, whose farmers have been most affected by fallout.

West Germany's present use of nuclear power is impressive. There are 19 nuclear plants producing 16,000 MW of electricity, or 36 per cent of the total. A further seven plants (including 300 MW Kalkar) are due to be commissioned by 1990, equivalent to 50 per cent of generating capacity. In North Germany, around Hamburg, no less than 70 per cent of electricity generation is nuclear.

Meanwhile, the SDP-Green coalition in Hesse has taken up the question of how to implement the Greens' demand that nuclear generation should be halted. A paper by the first regional environment minister (see *Nature* 321, 299; 1986), Joschka Fischer, accepts that there would be higher electricity prices and more air pollution during a transitional period, and has been called utopian by most politicians. But it won support last week from a surprising source, the director of the huge energy supplier Preussag Electra: a director, Hermann Kramer, said that the calculations are essentially correct.

Elsewhere, the CDU government of Schleswig-Holstein has delayed the start of the construction of the twentieth reactor planned at Brokdorf, near Hamburg, at least until after this week's elections in neighbouring Niedersachsen, while Ernst Albrecht has announced the creation of a new centre of solar energy research.

The federal government is softening its arguments. Chancellor Kohl is no longer asking for more nuclear power stations, while many SDU and FDP politicians suggest a "pause for reflection", while insisting that a halt to the nuclear programme would go too far and would not in any case make West Germany safe from nuclear accidents because there are at present a total of 66 nuclear plants in surrounding countries.

A reminder of that truth came last week, when the Oeko-Institut of Darmstadt announced that there had been a small release of radioactivity from its high-temperature reactor at Hamm-Uentrop, previously thought one of the safest. It seems that the leak was swamped by the fallout from Chernobyl, but the Nordrhein-Westfalen government has ordered the reactor closed until the company produces a full report on the safety of the system.

Meanwhile, the Bonn government has conceded one of the first demands of the Greens, and has appointed Walter Wallmann (CDU) as the federal government's Minister for the Environment, Nature Protection and Reactor Safety. The opposition regards the appointment as a clear admission of the mistakes of recent weeks. No issue has so much stirred West Germany as the fallout from Chernobyl.

Jürgen Neffe

US biotechnology

Row over industry regulation

Washington

CONGRESSIONAL frustration with the White House's delay over its regulation of the US biotechnology industry was plain last week at noisy hearings on Capitol Hill into Congress's own proposals on the subject. But Congress's attempt to act alone to regulate biotechnology was decried by administration officials and by industry representatives alike.

Although the administration's plans for coordinating agencies that deal with biotechnology were promised for early this

year, they had not appeared by the end of last week. The legislation proposed in the House of Representatives would upstage the administration's plans and make them irrelevant.

The chief proposals in the Biotechnology Science Coordination Act of 1986 are the establishment of a Biotechnology Science Coordinating Committee to scrutinize government actions, and also of a biotechnology science research programme to be paid for jointly by industry and by the government. The bill would

also modify statutes governing the Environmental Protection Agency and the US Department of Agriculture (USDA) to ensure that all environmental releases of genetically modified organisms that include DNA from different genera would be subject to special licensing and permit requirements.

The bill (HR 4452) was introduced by Representative Don Fuqua (Democrat, Florida), the outgoing chairman of the House of Representatives' Science and Technology Committee. Because Fuqua will not be present to lobby for the bill, it has little chance of becoming law in the current Congress. But congressional staff think that if a consensus emerges that new legislation is needed, there might be legislation next year.

The administration's proposals rely on existing statutes, and will exempt from special review those modified organisms whose new DNA includes only non-coding regions (see *Nature* 321, 458; 1986). The proposals have apparently been held up in the White House domestic policy council. David Kingsbury of the National Science Foundation, who directs the day-to-day business of the working group that drafted the guidelines, told an angry Representative Harold Volkmer (Democrat, Missouri) that he was "as frustrated as you are" over the delay.

The chairman, Representative James Scheur (Democrat, New York), even threatened to postpone the hearing until the administration had announced its plans. That draft copies of the administration's proposals have been leaked to industry spokesmen (admitted by the Industrial Biotechnology Association) did nothing to improve the congressman's temper.

One of the chief objections voiced to the bill was that a statutory Biotechnology Science Coordinating Committee would be less flexible than the existing committee of the same name, which consists only of senior officials of the relevant agencies meeting in private. The proposed new committee would have more open meetings, and industry spokesmen fear that it would be unable to deal with confidential business information in secrecy.

The proposed new biotechnology research programme was more popular, although industry spokesmen were worried that their newer members would be unable to afford such a scheme. The programme as outlined in Fuqua's bill would create a database bearing on regulatory decisions that would also point to projects for research. But witnesses were critical of limitations imposed on agencies' discretion to give special consideration to small environmental releases by research institutions, and said the cost of meeting unmitigated safety requirements might well prove prohibitive. **Tim Beardsley**

Chernobyl

Now for the long haul

THE Soviet government's special commission on the causes of the Chernobyl disaster and its aftermath will shortly be finishing its work, according to Academician Valerii Legasov, first deputy director of the Kurchatov Atomic Energy Institute at a Moscow press conference last week. But it has already been established, he said, that there are no grounds for abandoning the commercial use of RBMK reactors.

Not all participants in the press conference were so sanguine. Deputy Premier Yuri Batulin, in particular, called for "more time" to evaluate the disaster "comprehensively". He said that what was at stake was not simply a specific incident but the reliability of the whole nuclear power industry, a comment that tends to support an earlier statement of Academician Eugenii Velikhov that the safety systems of RBMK reactors still in service may have to be modified in the light of the commission's findings.

Time, however, is something the commission does not have if it is to retain its credibility and satisfy the demands of the planners. The optimistic tone of Soviet reports by engineers and servicemen mining under the reactor, dispersing radioactive rainclouds and spreading special decontaminating film have raised public expectations that life in the contaminated area will be back to normal within a few weeks.

By contrast, the experts are becoming more and more cautious. "Why risk your children's health by a premature return?" they urge. So, although the Ukrainian press has reported that the Chernobyl victims will have their names commemorated in new streets in the towns of Chernobyl and Prip'yat, it may be some considerable time before the memorial nameplates are unveiled. Instead of an early return home, the evacuated powerworkers and construction workers from the Chernobyl power station are to be

rehoused in new prefabricated estates on the outskirts of Kiev and Chernigov.

As far as farmland is concerned, Dr Yuri Israel, the head of the state commission for hydrometeorology and environmental control, says only that the "greater part" of the contaminated territory will eventually be "restored to the economy", implying that some areas may not be. A thorough study is now being made, Dr Israel told the press conference, of the potential doses that are involved in "protracted agricultural activity" in the affected areas.

Although (according to Batulin), the long-term economic effect of the close-down at Chernobyl will be "barely perceptible" in the statistical reports, it seems that the shortfall in power generation has been covered only by using all other Ukrainian power stations at full capacity. This is clearly a short-term solution, and it has been officially stated on several occasions that the three remaining Chernobyl reactors will be back in operation in the autumn.

In spite of the use of robots and remote-control digging machinery, there are still considerable health constraints on prolonged work at the site itself. Moreover, the references last week to a new phase of laying up the damaged reactor and the long-term measures to prevent seepage on contaminated ground water suggest that many of the practical details still remain to be worked out.

Meanwhile, new problems continue to occur. A number of wells in Byelorussia have been found to be contaminated (whether through seepage or rainfall is not clear), while forest fires in Poles'ye have begun recirculating the radioactive dust. In spite of earlier assurances that Kiev would remain safe from water-borne pollution, the public have now been warned against bathing in the Dnieper or relaxing on the river-beaches until further notice.

Vera Rich

French science

Old guard takes high jump

IN a week of long knives, the new right-wing government of France has removed three of the old guard in charge of the major scientific institutions. The development is to say the least surprising, coming as it does just a few weeks before the council of ministers has to decide the distribution of a FF40,000 million (£4,000 million) cut in overall government spending.

Last Wednesday, Pierre Papon, director-general of the Centre National de la Recherche Scientifique (CNRS), with its 20,000 staff and budget of FF9,000 million (£900 million), was sacked. Independently, Jean-Jacques Payan, in charge of the universities, and Bernard Descombes, who had introduced innovative new funding structures designed to improve university research, resigned.

The sacking of the CNRS director-general, who was re-appointed by presidential decree for a three-year term only last December, is unprecedented. Papon had been a key figure in the development of the previous administration's policy on science, and his removal may be symbolic of the new government's determination to have its way. Usually (as with Payan and Descombes) the government is content to provoke the resignation of those among its senior officials with whom it is out of sympathy.

Papon seems to have been determined to hold on to protect the considerable achievements of the old regime, and had been planning in September to present a package of policies designed to achieve that to the new science minister, Alain Devaquet. But the new government seemed unable to wait.

Nevertheless, Papon's replacement should not cause too many shudders in the scientific community, at least if he inherits some of his predecessor's power. The new man, Serge Feneuille, is a reputed physicist who for five years has been chief scientist of the cement makers La Farge Coppée, a company which with Feneuille's help had begun to diversify and take an interest in biotechnology.

Despite the pre-election pressure from the right to dismantle the vast and interdisciplinary CNRS, the feeling at the research council, where Feneuille has worked before, is that he will "protect" the institution. With his background, he should also be interested in continuing to forge links between the scientific community, industry and the public in line with the previous administration's policy.

Such doubts as there are arise because of the government's budget-cutting mood and the apparent political weakness of the research minister Alain Devaquet, a man whom most scientists are willing to em-

brace as an ally against a larger foe, the finance ministry, which has emerged as the main instrument of Prime Minister Chirac's political philosophy. The outlook for research spending in France looks bleak, with a new CNRS director-general reporting to a minister already in a weak political position and with major budget decisions due in July.

Sacked Papon spoke last week of the need to maintain the integrity of CNRS,

and especially, the momentum of the interdisciplinary programmes on which he spent much of his effort. "Feudalities are a permanent temptation in France", Papon said.

He is also anxious that the policy of cooperation between universities and industry should be maintained. "The end of the Ministry of Research and Technology was a mistake", says Papon, because of the separation of "responsibilities for science and technology" which is implied. If the ministry is not strong, "it will be the end of the coherence of the whole programme".

Robert Walgate

US policy

Another new White House man

Washington

PRESIDENT Ronald Reagan last week nominated William R. Graham, deputy administrator of the National Aeronautics and Space Administration (NASA), as his science adviser and director of the White



House's Office of Science and Technology Policy (OSTP). The twin posts had been without a permanent occupant for the past 6 months, since George A. Keyworth II resigned in December to form a private consulting company. The acting director of OSTP since the start of the year, John McTague, resigned two weeks ago to take a position in industry.

The six-month delay in finding a replacement for Keyworth has provoked criticism from the scientific community. Several potential candidates have turned down offers, and many observers felt the difficulty in finding a permanent incumbent was indicative of the diminishing influence of OSTP in the White House. Several other senior OSTP officials have resigned in recent months without being replaced, and the office's proposed budget for 1987 is a hefty cut from this year's level.

Graham's nomination has therefore been greeted with subdued relief by scientific leaders, but some express reservations that his military background and advocacy of the militarization of space made him a less than ideal candidate. Robert Park of the American Physical Society said that the appointment was

consistent with Reagan's previous appointments of "technocrats" to the post, rather than of distinguished academic scholars.

William Carey, executive officer of the American Association for the Advancement of Science, who had been vocal in his criticism of the hiatus at OSTP, said he was glad someone was now in place who was familiar with high-technology initiatives. But he admitted to being concerned that Graham's background might lead him to offer advice on investment decisions that would be "tilted in one direction". Asked specifically about Graham's known support for the Strategic Defense Initiative, Carey responded "what's new?". Keyworth was also a staunch defender of the programme.

Graham first came to public attention last year when he stepped in to become acting administrator of NASA after the indictment on fraud charges of NASA's former administrator, James Beggs. With the appointment in May of James Fletcher as permanent NASA administrator, Graham needed somewhere to go, as Fletcher had no special desire to keep Graham on as his deputy. Graham's appointment will need confirmation by the Senate, but because his appointment at NASA was also the subject of a confirmation hearing, no serious opposition is expected.

Before going to NASA, Graham was chairman of the general advisory committee of the Arms Control and Disarmament Agency. He started his career as a research physicist at Stanford University, and subsequently worked on survivability of strategic systems for the Air Force and a National Research Council study of submarine warfare. In 1971 he formed a consulting company, R&D Associates, and he has frequently been a consultant for the Department of Defense. But there was some criticism of his performance at NASA during the admittedly turbulent period of his office as acting administrator.

Tim Beardsley

Laboratory accidents

Inquiry into lab's bone cancers

THE Institut Pasteur in Paris has set up an external commission of inquiry to investigate the occurrence of three cases of bone cancer among workers in the same laboratory at the institute. Two of those affected have died in the past few months, and some colleagues suspect that the cluster of cases may be linked with work on oncogenic viruses being carried out in the laboratory. The outcome of the inquiry will be awaited anxiously by those elsewhere whose research involves mammalian oncogenes.

The Pasteur laboratory is called the genetic toxicology laboratory, and is largely concerned with the molecular biology of oncogenic viruses and of oncogenes. One of those who has died, Dr Françoise Kelly (aged 50), had worked in the laboratory for four years. A close friend of Dr Kelly's says that, before her death, she had come to believe that there should be an investigation of the hazards of the research on which she had been engaged.

Most recently, Kelly had been working on a project to effect the transformation of mouse embryo carcinoma (teratoma) cells by the transfer of genes derived from viruses known to be oncogenic in certain circumstances, simian virus 40 (SV40) and adenovirus in particular.

Specifically, Kelly was attempting to endow the mouse cells with a synthetic gene consisting of the "early T" gene of SV40, the DNA coding for a protein produced early in the viral life-cycle, linked with the promoter region of an early gene from adenovirus.

According to a spokesman at the Pasteur Institute, the experimental manipulations were carried under a hood which, while drawing air from the surrounding experimental area, filtered it through active charcoal. The spokesman added that the procedures followed in the genetic toxicology laboratory were "much more careful" than those common in mo-

lecular biology laboratories because members of the same staff were also used to dealing with a stream of potential industrial and environmental mutagens and carcinogens.

Little is known about the mutagens and carcinogens handled in the laboratory, apparently because much of the work is confidential to the commercial companies and other interests by whom it has been commissioned. According to an institute report in 1981, it signs secrecy agreements with clients who commission testing. It is, however known that the laboratory has worked on derivatives of the benzo and naphtho-nitrofurans, known to be powerful mutagens.

The possibility that the cluster of three bone cancers may be linked with the programme of testing for mutagenicity and carcinogenicity is contradicted by the usual length of the latency period for cancers induced by chemicals.

The second researcher to die, aged under 40, had worked in a laboratory across a corridor from Dr Kelly, but only for a period of six months in 1979-80, before the project to transform mouse embryonic cells had been begun. The third bone cancer patient, who is at present ill, is also under 40, and had worked in the same room as Dr Kelly although on a series of genetic manipulations of *E. coli*. There is no sign of damage to the technicians and the two students who had been assisting Dr Kelly with her last project.

These circumstances lend support to the suggestion by the director of the Pasteur

Institute, Raymond Dedonder, that the occurrence of the three cancer cases is probably a statistical coincidence. Dedonder put this view at a private meeting with Pasteur staff earlier this year. And while it seems that one of the bone cancers is a primary osteosarcoma, it appears that Dr Kelly's bone cancer was metastatic, and that the character of the primary cancer has not, in her case, been determined.

The case that the cluster may be statistical in origin is further supported by what is known of the movements of those involved. During the past ten years, between 200 and 400 people have worked in the Pasteur laboratories in which the three cases of bone cancer have now been recognized. Senior members of the Pasteur Institute also point to the occurrence of a similar cluster of cancer cases (brain cancers, characterized as glioblastomas) at a laboratory at Orsay over a period of twelve years; surprisingly, this has been shown to be consistent with chance.

Arguments such as these appear to have dampened most fears at the Pasteur itself, but there remains some concern that the external committee of inquiry set up by Dedonder last month may be inclined to cloud the issues. The commission includes Professor Jean Bernard, who is president of the French national ethical committee which considers issues raised by the new biology, and Professor Maurice Tubiana, director of the Parisian cancer hospital at Villejuif, both respected figures in French medicine. But the commission also includes three members chosen by the Pasteur's safety committee, a long-standing thorn in the flesh of the French nuclear industry.

Robert Walgate

Foreign students in science and engineering

Of all technical disciplines, engineering has the highest proportion of foreign students in each of the five countries above. The proportion ranges from 28 per cent in West Germany to 21 per cent in Japan. Japan also has the lowest percentage of foreign students in natural sciences (3 per

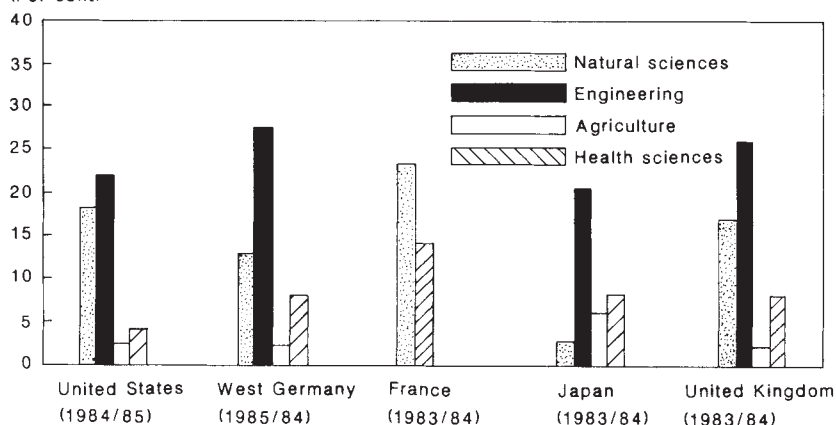
cent), compared with 18 per cent in the United States. For France in the histogram there are no data available for engineering, and agriculture and natural sciences are combined. From International Science and Technology Data Update 1986, National Science Foundation, Washington, DC. □

Chernobyl fallout: corrigendum

In the letter "Initial observations of fallout from the reactor incident at Chernobyl" by L. Devell *et al.* (*Nature* 321, 192-193; 1986), the inventory figures in the first column of Table 2 are low by a factor of 2.92. The correct column should read:

Nuclide	Inventory (10^{17} Bq)
^{137}Cs	1.52
^{131}I	29
^{106}Ru	41
^{141}Ce	53
^{132}Te	41
^{239}Np	543

(Per cent)



AIDS virus nomenclature

SIR—Since July 1984 I have been writing on acquired immune deficiency syndrome (AIDS) for the London weekly free newspaper, *Capital Gay*. In the issue of 11 April I referred to a rumour that “human immune deficiency virus”, “HIV” or “HIV”, was about to be proposed as the name for the causative agent. I fear that Robert Gallo is therefore mistaken in attributing first publication of this nomenclature to Luc Montagnier.

Your own objection to “HIV”, that the “IV” might be confused with the roman “four”, could be resolved by adopting the abbreviation “HIDV”, for which there is an obvious precedent in “AIDS” for “acquired immune deficiency syndrome”. However, combinations such as “HIV infection”, “HIV disease”, “HIV brain disease”, “HIV lung disease” and “HIV lymphadenopathy” read far better than their “HIDV” equivalents.

As for the idea of “HTLV-III/LAV”, fossilized forever, forget it. Are we expected to continue with “HTLV-IV/LAV-3” and “LAV-2/HTLV-V”? What next?

While the name of the virus is totally irrelevant to the needs of those directly affected by it, an agreement to use the new name opens the way to an easily-understood terminology for the whole spectrum of conditions linked to this virus, which would be very useful to the agencies concerned with educating the public and health professionals about it.

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SIR—At a meeting on 22 and 23 May 1986, the Executive Committee of the International Committee on Taxonomy of Viruses (ICTV) endorsed the name human immunodeficiency virus, recently proposed by a large majority of the members of a study group of ICTV, headed by Harold Varmus (J. Coffin *et al. Nature* **321**, 10; 1986 and *Science* **232**, 697; 1986), as appropriate for retrovirus isolates implicated as causing acquired immune deficiency syndrome (AIDS). The new name describes the host and a major biological property of the virus, recognizes the difference of the virus from isolates of human T-cell lymphotropic virus types I and II and avoids any controversy regarding priority of discovery of the virus and emotive connections of the virus with AIDS and its methods of transmission.

ICTV is working towards a uniform international nomenclature for viruses based on their taxonomy. Much still remains to be learned about the relationships of the human immunodeficiency virus with other retroviruses and therefore designation of an international name

would be premature. However, the committee recommends the use of the name human immunodeficiency virus as the vernacular name to replace HTLV-III and LAV.

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French science policy

SIR—France may be one of the richest countries in the world, but prosperity does not uniquely determine a country's scientific potential. The recent developments in science policy in France, and in particular the emphasis on applied sciences, is not a particularly creditable achievement for the policy-makers.

Every laboratory now seems to have been diverted towards applied science. In biology, chemistry, physics and mathematics, it is almost as if those responsible for policy have concluded that France has now done enough in basic science, and the time has come to exploit this knowledge for practical purposes. The result is debilitating for the laboratories previously concerned with basic science, now compelled to work in applied science to conform with the present fashion.

There is also a clear difference between the way in which those supported by the Centre National de la Recherche Scientifique (CNRS) establishment are equipped and the provision for ordinary academic scientists, who often work in the same laboratories. While CNRS groups have by no means everywhere attained the targets they have been set, they are nevertheless pampered with generous grants and sophisticated facilities that do not exist in ordinary university laboratories. Why should there be such a segregation?

In the basic science laboratories of the universities, scientists are to be found working on applied projects simply to earn the funds needed for their survival and to remain among the ranks of active scientists. No thought seems to have been given to this problem.

At the same time, research students are busily accumulating theses, a waste of money and time. These students are better served if they are trained in first-class laboratories instead of being fed a huge bulk of theoretical science. Policy-makers would find that if they abolished the present system, they could save time and money that could be used partly to safeguard French basic science.

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Chernobyl's message

SIR—An important lesson of the Chernobyl disaster is that “probabilistic risk analysis” is a misapplied science. Its irrelevance to decision-making is now amply demonstrated. A core meltdown, designed to be less than a “one-in-a-million” chance in all reactors adopted by members of the International Atomic Energy Agency, has occurred twice within a decade.

Although such mathematical tools have their use in reactor design, they are still being applied uncritically in technology policy. In this respect they are dangerous, as they divert attention from the essential safety issues. These concern the hazard once realized (however improbably): control, and communication with and protection of the affected public. Doubtless misled by their own reassuring probabilistic predictions, Soviet scientists were caught unprepared in all respects, like their US colleagues at Three Mile Island.

British reactors are, on paper, exceptionally safe compared with Soviet reactors. But can the British public now believe that there is no need for detailed and well-rehearsed contingency plans for nuclear disasters? These should include not merely technical procedures on site, but also tested plans for communication with the public, and evacuation procedures for at-risk populations.

Developing such plans would involve scientists, public authorities and communities, in an extensive, open consultative dialogue. In the absence of such a programme, the nuclear industry's credibility will not be rescued by more reassurances couched in statistical rhetoric.

S.O. FUNTOWICZ

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No chance...

SIR—As positive probabilities refer to events that can happen, negative probabilities presumably refer to events that cannot happen. The greater the probability that the event cannot happen (as distinct from being unlikely to happen), the higher the (negative) numerical value.

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Erratum

IN the letter “Too many fourth states of matter?” by J.M. Goldschvartz (*Nature* **320**, 302; 1986), the second sentence in paragraph 2 should read: “Moreover, physicists should be aware that since 1940 the attribution was used to describe the superfluidity of liquid helium 4 and 3 (refs 3–6)”. □

Towards understanding snowflakes

The reasons why the microscopic anisotropy of molecules in crystals is reflected in the shapes of the macroscopic structures which they form have been obscure. Now there is some progress.

THE celebrated symmetrical hexagonal dendritic shapes of snowflakes are so familiar that they should long ago have been explained; that might be everybody's reasonable expectation. But the physics of the problem is not nearly as simple as it seems. The essential difficulty is to account for the way in which anisotropy on the scale of molecules is reflected in the shapes of macroscopic crystals.

The problem is not especially difficult with crystals that form a reasonably compact mass. The crystals grown by the multitude in kindergarten classrooms by suspending a crystal seed by a piece of thread in a supersaturated solution form in regular shapes for energetic reasons, because that is how best to minimize the free energy of the system; provided that the access of extra molecules (or ions) is equally easy from all directions, which requires that in practice the crystal seed should not be too close to the side of the jar in which it is suspended, extra elements will be added in such a way as to form sheets parallel to the planes in the basic unit cell. If access is not independent of direction, the result will be a misshapen crystal, but the underlying symmetry will remain at the level of the unit cell.

Dendritic structures such as snowflakes are more difficult because they combine what often seems a startling gross symmetry with a disconcerting disorderliness on the scale of the dendrites. The simple-minded rules of crystal growth seem not to apply. The article by Johann Nittmann and H. Eugene Stanley (this issue, p.663) goes a long way to explain why some recent attempts at a solution have not succeeded fully. More than that, it embodies the essence of an explanation of the growth of dendritic crystals.

Broadly speaking, there have been two complementary approaches to the problem, the first due to J.S. Langer (reviewed by Langer in *Rev. mod. Phys.* **82**, 1; 1980), who has dealt with the growth of dendritic tips by the diffusion of new material through the surrounding medium. The arguments apply as well to crystallization from a solution as to condensation from a vapour phase. The rate at which material is added to a particular point on a growing surface is determined by the local geometry in the sense that protuberances are more likely than re-entry points to acquire material by diffusion. There may be circumstances in which growth is limited by the need that latent heat should be con-

ducted away from the surface. The fact that growth depletes the local environment of the uncondensed phase of material means that growth will also be limited by the rate of diffusion. The usual upshot is a set of differential equations that cannot usually be solved exactly, but can be used to define the conditions under which a dendritic tip will continue to elongate or, alternatively, split.

The frustrations of this approach have stimulated the more recent phase of computer simulation, of which T.A. Witten was the first exponent (see Witten, T.A. & Sander, L.M. *Phys. Rev. Lett.* **47**, 1400; 1981). In the simplest model of diffusion-limited aggregation, as it is called, extra elements of the material of which a crystal is being constructed are supposed to be added to the system at random points of some distant boundary and are allowed to migrate by means of a random walk along the links of some underlying lattice. If the diffusing element should reach a point on the lattice which is an immediate neighbour of the growing aggregate, it may be supposed captured, but there are a host of ways of defining the aggregation rules, most of which appear to have been tried in the past few years.

The virtue of these simulations is that they yield patterns which indeed resemble those of real aggregates. In particular, they show that aggregates whose growth is limited by diffusion are fractal structures; in two dimensions, for example, the number of elements in an aggregate is not proportional to the square of its dimensions but to some lesser power of them, called the fractal dimension. At some cost in computer time, the simulations can be (and have been) carried out in any number of dimensions.

What Nittmann and Stanley have done is to combine these two approaches or, more accurately, to carry out lattice-based simulations of aggregation in a way that can be made to correspond to equations of the kind that Langer (and, now, many others) formulated. They have overcome the persistently annoying feature of simulations, the difficulty of telling the meaning underlying the patterns produced, by producing a series of patterns corresponding to the variation of some physical parameter over a known range. Their aggregation rules are among the simplest there could be, consisting simply of the requirements that new elements are added at random to the periphery of a growing

aggregate, but that their assignment to the sites available is biased to reflect what the macroscopic equations suggest will be the rate of growth there.

One result of consequence to emerge arises from the fact that the simulation model can deal only with the addition of discrete elements to the aggregate. There are, as a consequence, departures from the macroscopic prediction of the regular outward extension of a regular envelope of the growing aggregate, which is fairly described by the authors as noise. The essence of this part of the result is that outward extensions of the surface are quickly smoothed out by the later addition of material, but that inward depressions are less readily filled in, but may instead persist as the apices of what are to become fiords in the pattern as the aggregate grows.

The simulations (see p.664) show that the patterns are typically broken up into a number of radially extended tongues of substance separated by lengthening fiords, while the tips of new fiords repeatedly form at the outer surfaces of the extending tongues. Given that the aggregation rules correspond to the penetration of one liquid into another with which it is immiscible, it is pleasing but not surprising that the fractal patterns formed resemble those found in such experiments. The circumstances correspond to those of practical importance in which oil companies pump water down into the oil wells so as to displace crude oil from its reservoirs.

But the most remarkable result of these simulations stems from the apparently successful attempt to allow for the anisotropy of the real world, which depends on the assumption that not all sites on the surface of a growing aggregate are equally suitable energetically. The criteria used in extending the aggregation rules are strictly microscopic; they depend only on the orientation — some sites are more likely to be occupied than their immediate neighbours.

One outcome is the pattern of an aggregate which is virtually indistinguishable from a fairy-tale snowflake, a feathery collection of dendrites which is plainly hexagonally symmetric overall, but whose smaller-scale structure is far from symmetric, which is the case with real snowflakes. That looks a convincing demonstration that the snowflake problem is on the way to solution.

John Maddox

Molecular biophysics

Energy and signal transduction by transmembrane protein complexes

from Alexander N. Glazer

A RECENT conference* brought together molecular biologists, biochemists, X-ray crystallographers and spectroscopists to discuss the structure and function of various transmembrane protein complexes. These molecular machines enable cells as well as intracellular organelles to generate proton and ion gradients, transport metabolites, transduce information from one side of the membrane to the other and to control flagellar motion. But details of the mechanisms whereby these functions are accomplished remain to be discovered.

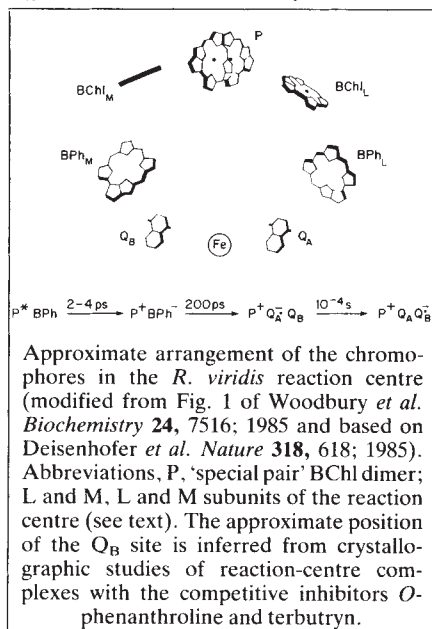
Approximately two years have passed since the determination of the crystal structure of the reaction-centre complex from the purple bacterium *Rhodospirillum rubrum*. This achievement provided the first (and to date the only) high-resolution view of an integral membrane-protein complex. The *R. rubrum* reaction-centre complex contains four molecules of bacteriochlorophyll-b (BChl), two of bacteriopheophytin-b (BPh), two different quinones (menaquinone, Q_A and ubiquinone, Q_B), a non-haem iron and four haems. All these components are embedded in a complex of four polypeptide subunits. (See Henderson, R. *Nature News and Views* 318, 598; 1985 for further explanation.)

Electron flow

In principle, it should be possible now to gain a precise understanding of the details of electron flow through the complex, and the first session at the meeting dealt with studies directed towards that end. The formation of the P^+BPh^- state takes place within 4 picoseconds of excitation of the special pair of BChls (see figure). Topological considerations suggest that the electron might flow through the BChl which is between the special pair and the BPh. But P^+BChl^- does not appear to be a resolvable intermediate in the electron-transfer sequence. A P^+BChl^- charge-transfer state facilitates electron tunnelling between P^+ and BPh (see figure) by mixing electronically with both P^+ and P^+BPh^- (W.W. Parson, University of Washington).

The protein environment of the prosthetic groups modulates their properties in important ways. For example, electron paramagnetic resonance and electron-nuclear double resonance (ENDOR) data

(W. Lubitz, Freie Universität, Berlin) on the *R. rubrum* P_{960}^+ radical cation indicate an asymmetrical spin distribution. Moreover, the two BPh groups are spectroscopically distinct — the electron travels selectively down the pathway directed towards the Q_A site. A major feature of the reaction centre is that Q_A accepts one electron whereas Q_B can be doubly reduced. Both semiquinones are bound to the protein through hydrogen bonds. For Q_B^- , these bonds (of length r) appear to be symmetrical and fairly long whereas for Q_A^- the ENDOR data clearly indicate two



hydrogen bonds of different strengths, about 1.3 and 1.6 Å, respectively. The asymmetrical H bond at the A site might be important in adjusting the redox potentials and locking Q_A as a one-electron gate.

Attempts to elucidate the biosynthesis, assembly and function of purple bacteria reaction centres and antenna complexes using molecular genetics are also in full swing. Thus far genetic manipulation has been confined to the photosynthetic apparatus of *R. capsulata* (now called *Rhodobacter capsulatus*). Replacement in the L subunit of a special-pair BChl ligand, His 174, by Leu leads to a loss of all three reaction-centre subunits from the membrane. But both the light-harvesting LHI and LHII antenna complexes assemble normally (D.C. Youvan, Cold Spring Harbor Laboratory).

Another surprising result was reported by F. Daldal (Cold Spring Harbor Lab-

oratory). It had been thought that in *R. capsulata* cytochrome c_2 served as an obligate electron carrier between the cytochrome bc_1 complex and the reaction centre. Daldal replaced a portion of the cytochrome c_2 gene encoding the haem-binding domain of the protein with an insert encoding kanamycin resistance. As expected, the mutant cells contain no cytochrome c_2 . The unexpected result is that the mutant grows at near wild-type rates under photosynthetic conditions.

Spectroscopic studies of electron transfer in both mutant and wild-type cells prompted by these findings reveal a complex situation (R. Prince, Exxon Corporation). In wild-type cells, the cytochrome c_1 of the bc_1 complex delivers energy directly and rapidly with a half-time of less than 100 μs to about 20 per cent of the reaction centres. A further 20 per cent receive energy rapidly from cytochrome c_2 , whereas 30–40 per cent receive electrons on a timescale of several milliseconds. The slow transfer is attributed to cytochrome c_2 functioning as a mobile carrier diffusing along the membrane-aqueous interface and shuttling electrons from the cytochrome bc_1 complex to more distant isolated reaction centres.

Cytochrome complex

The ubiquitous cytochrome bc_1 complex was itself much discussed. In photosynthetic organisms, it stands at the crossroads of respiratory and photosynthetic electron flow. Independent of source, the bc_1 complex contains a cytochrome c_1 , a cytochrome b with two haem centres and an iron-sulphur protein. In the mitochondrial complex described by G. von Jagow (University of Munich), the Fe_2S_2 protein of relative molecular mass (M_r) 21,600 (21.6K) is largely polar and is attached to the outside of the mitochondrial cristae membrane by a short anchor at the carboxy terminus. Cytochrome c_1 (M_r = 28K) is also a polar protein with a membrane anchor inserted into the cytosolic surface of the membrane. Cytochrome b is a transmembrane protein (M_r = 42.4K) with a haem b_{566} centre (oxidation-reduction midpoint potential E_M = -50 mV) at the cytosolic surface of the membrane and a haem b_{562} centre (E_M = +50 mV) near the centre of the membrane. The iron atoms of the two haem b groups are spaced some 20 Å apart. The two haems create a path for electron transfer across the membrane. Six different small subunits, all of unknown function, are associated with the three polypeptides bearing the four redox centres. In contrast, the *Paracoccus denitrificans* bc_1 complex has the simplest composition and one of the highest turnover numbers of any bc_1 complex isolated to date (B. Trumpower, Whitehead Institute). The complex is made up of three polypeptides:

* The Banbury Conference Microbial Energy Transduction: Genetics, Structure and Function was held at the Cold Spring Harbor Laboratory on 23–26 February 1986. A summary will be published shortly by the Cold Spring Harbor Laboratory.

cytochrome c_1 ($M_r = 62K$); cytochrome b ($M_r = 39K$); and the Fe_2S_2 protein ($M_r = 20K$). The much larger size of the cytochrome c_1 polypeptide suggests that its sequence encompasses some of the components found as individual polypeptides in mitochondrial bc_1 .

The notion that bacterial complexes are generally simpler than their mitochondrial counterparts was supported further by a comparison of *Escherichia coli* cytochrome d terminal oxidase (R.B. Gennis, University of Illinois) with the cytochrome oxidase of mitochondria (T.G. Frey, University of Pennsylvania). The former complex is made up of one copy of cytochrome c_1 and two of cytochrome d , whereas the mitochondrial complex contains six to twelve different polypeptides. Structural information on two-dimensional crystals of the mitochondrial complex is available at a resolution of 20–30 Å, but the question of whether mitochondrial cytochrome oxidase is a dimer or monomer *in vivo* remains unanswered.

In the examples described above, the distinctive spectroscopic properties of the multiple prosthetic groups provide direct access to information bearing on the consecutive steps in the electrogenic reactions which these complexes perform. Unfortunately, this is not the case with the next group of transmembrane complexes discussed — for example, the H^+ -translocating ATP synthases, ($Na^+ + K^+$)ATPase and Ca^{2+} ATPase. Consequently, speculation still occupies centre stage in proposals for the detailed mode(s) of action of these complexes.

Common mechanism

There is increasing evidence that all of the synthases which react with ATP to form a covalent β -aspartyl phosphate (D^*) intermediate may share a common mechanism of action. For example, the sequence CSD*KTGTLT (single-letter code) is fully conserved in the H^+ ATPase of *Neurospora* and *Saccharomyces cerevisiae*, the ($Na^+ + K^+$)ATPase of sheep kidney and the Ca^{2+} ATPase of rabbit muscle. Moreover, a 15-residue sequence near the carboxy terminus of these enzymes shows variation in only three positions. The sequences of all three enzymes give rise to very similar hydrophathy plots (C.W. Slayman, Yale University). In contrast, enzymes of the bacterial phosphotransferase system, enzyme II^{pm} and enzyme II^{pm} –enzyme III^{pm} , which phosphorylate and transport mannitol and glucitol, respectively, show no significant sequence homology (M. Saier, University of California, San Diego).

R.M. Macnab (Yale University) presented the latest chapter in the continuing saga of studies of the bacterial flagellar motor. Genetic and biochemical studies have focused on five polypeptides which are not integral components of the basal

Robert Day Allen (1927–1986)

ROBERT DAY ALLEN, who died at home on 23 March after an intense one-year fight with pancreatic cancer, was one of the modern founders of the field of cell structure and motility, an area that he investigated during his entire career.

After a doctoral dissertation under L.V. Heilbrun on the nature of artificial activation of clam eggs, Allen continued basic studies on the structure of egg cytoplasm and pronuclear movements at the Wenner-Gren Institute, the Kristinebergs Zoological Station in Sweden, followed by a period at the University of Michigan and then at Princeton University. It was during this period that he evolved two major concepts that stayed with him during his entire research career. One concept was that living cells could yield important information about basic biological processes; the other that light optical methods in concert with electronic control and detection devices could yield molecular information in living cells. Allen was clearly one of the pioneers in applying quantitative light microscopy to basic questions in cell and molecular biology.

He next combined the use of biophysical techniques, especially light microscopy, to test the prevailing dogma on the mechanism of amoeboid movement. A combination of studies on living cells and isolated cytoplasm led him to re-examine the tail contraction model. He demonstrated that cytoplasm was not a newtonian fluid and possessed structure as evidenced by its optical polarization properties and ability to transmit applied tension. His observation, in 1955, that isolated cytoplasm from single cells could continue to generate motile forces ultimately led to the preparation of motile cell extracts that continue to yield important biochemical information today.

This early work demonstrated, experimentally, two fundamental concepts that had been discussed previously with much less experimental evidence: first, that cytoplasm can exist as both a medium of weak structural properties (sol) and as a viscoelastic solid (gel); and second, that the elements responsible for the generation of contractile forces exist in the cytoplasm. Allen's research on the structure and motility of cytoplasm led him to propose the frontal contraction model of

amoeboid movement, still used as a valuable guide in the design of experiments for testing mechanisms of this type of movement.

In the late 1960s and early 1970s, at the State University of New York at Albany, Allen assembled his first electro-optical microscope. The phase-modulated microspectrophotometer enabled him to measure separately the optical polarization properties of linear birefringence, linear dichroism, optical rotation and circular dichroism in tissue sections, muscle fibres and solutions of proteins.

The last part of Allen's career was probably the most important in that he had a seminal influence on the development and use of video-enhanced contrast microscopy in cell and molecular biology. This method allows large increases in optical contrast which facilitates the detection of structures well below the limit of resolution of traditional optical microscopy. At the Marine Biological Laboratory in Woods Hole, Massachusetts, Allen and his many collaborators investigated fast axonal transport in squid axons and in isolated axoplasm. The fundamental discovery was that particles are transported along single microtubules. Furthermore, individual microtubules can exhibit motility along glass substrates. This exciting discovery has stimulated research in several fields where microtubules have been implicated in motile events.

Robert Allen loved the challenge and thrill of performing basic research and communicating the results. He was particularly interested in seeing the same kind of enthusiasm and drive in students. A major part of his interest in science was in communicating facts and concepts in novel ways. He produced many educational movies and pioneered the use of video disks as a medium for publishing results in the journal *Cell Motility* which he founded. His uncanny intuition in both biological processes and in optical methods was expressed repeatedly in his work on the structure and motility of cytoplasm, organelles and whole cells. The fluid nature of his scientific thought and experimental approach was undoubtedly influenced, in part, by his abilities as a concert cellist. His extraordinary talents will be missed, but his impact will long be felt. D. Lansing Taylor

body. Two of these, MotA and MotB, appear to be circumferentially arranged about the basal body and involved in interaction with other components of the motor to transduce proton potential into torque. The rest, Fla AII.2, FlaQ and FlaN, are important for assembly, rotation and switching. Deletion mutants in the corresponding genes do not assemble any detectable flagellar structure. Mis-

sense mutants, depending on the allele, may be Fla⁻, Mot⁻ or Che⁻. The available evidence suggests that these switch proteins form a complex that is peripheral to the membrane and may be associated with the M-ring of the basal body. □

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Mammalian energetics

Energetic costs of reproduction

from Paul H. Harvey

How expensive is reproduction? The answer depends on how we measure costs. Many evolutionary biologists and behavioural ecologists think in terms of parental investment, which is defined as decrements in a parent's future reproductive success that result from a current reproductive episode¹. In contrast, ecologists and physiologists tend to focus on energetic costs which can be measured during pregnancy and lactation, either directly as energy allocated to reproduction or indirectly by increases in metabolic turnover or loss of fat reserves. A recent meeting* showed how advances in technology coupled with an ingenious choice of subjects for study provide a useful database which answers some questions but poses many more². One of the most interesting results presented is reported by S.D. Thompson and M.E. Nicoll on page 690 of this issue³.

Particularly striking contrasts in both reproductive patterns and energy needs are apparent when marsupials are compared with other (eutherian) mammals⁴. Large-bodied mammal species wean large offspring. A head start is given to the young of many eutherians because large species have long gestation length and produce large neonates. This trend is not so evident among the marsupials (gestation length is independent of maternal size, and the increase in neonatal weight with maternal size is much less marked). Furthermore, for their body sizes, marsupials tend to have lower basal metabolic rates than do eutherians, yet they still manage to wean larger offspring. How, then, do marsupials cope with the energy demands of lactation? The simple answer, illustrated by the work of Thompson and Nicoll³, is that productive females of at least one species of marsupial (and two eutherian mammals with low metabolic rates) increase their resting metabolic rates during gestation and lactation to about the normal eutherian level, whereas the typical eutherian barely increases its higher resting metabolic rate. Which is energetically the more costly mode of reproduction — the short gestation and long lactation of marsupials, or the typical eutherian pattern of long gestation and shorter lactation? We do not yet know.

Size and taxonomic affinity are not the only correlates of basal metabolic rate. For several years, Brian McNab (University of Florida at Gainesville) has been

pointing out dietary associations with metabolic rate⁵⁻⁷. Among the eutherian mammals, species feeding on invertebrates, fruit, seeds or the leaves of woody plants have low metabolic rates whereas those feeding on vertebrates, nuts and herbs tend to have high rates. McNab postulates cause and effect relationships, arguing that eutherian mammals exhibit the highest metabolic rates that their diet will allow, because high rates permit high reproductive output⁶. Species with low metabolic rates, so his argument runs, live on foods that "limit the rate at which energy is acquired by a mammal". For example, leaves of woody plants and some



Does the mass-specific resting metabolic rate of all marsupials increase during lactation? Bennett's wallaby (*Macropus rufogriseus*) with young, currently being studied at the Zoological Society of London.

invertebrates have low digestibility and often contain chemical deterrents to herbivory or predation. Such arguments may, of course, amount to nothing more than adaptive story telling, and might best be viewed as hypotheses that need testing.

How do the low mass-specific basal metabolic rates of the marsupials fit into McNab's scheme? The alternatives are that marsupials never live on diets which allow high rates of energy acquisition, or that increased metabolic turnover does not result in higher reproductive output among marsupials. Marsupials differ considerably in their diets, and like their eutherian counterparts, frugivorous and folivorous marsupials have low metabolic rates. However, vertebrate- and nut-eating marsupials do not have high metabolic rates. This, McNab claimed at the meeting, is because high basal metabolic rates are not reproductively advantageous for marsupials. His statement is supported by an apparent positive correlation between

the maximum rate of population increase and basal metabolic needs in eutherians and the absence of such a correlation in marsupials. McNab's claim needs qualification in the light of Thompson and Nicoll's new work, and does not appear to have a ready explanation.

Metabolic rates like many other physiological and reproductive variables, change with body size. Almost all the work reported at the meeting included routine allometric analyses, which can place many problems into clear perspective. T.H. Kunz, A. Kurta and E.D. Pierson (Boston University) reviewed recent work showing that bats invest heavily in reproduction. Neonates are more than 20 per cent of the mother's body weight in most families, and even reach 38 per cent in the Rhinolophids. Is this unusual for a mammal? It is, but bats are small mammals, and small mammals produce disproportionately large young. When body size is taken into account, bats do produce large neonates but litters are small (generally singletons), so that litter mass is typical for a mammal the size of a bat. Why, then, do bats produce large single young? Bats wean when they are relatively young, perhaps an important feature in such mobile mammals. If the production of large young is an evolutionary tactic to shorten dependence on the parents, a trade-off between prenatal and postnatal development is implied. Yet the opposite pattern is found in primates and birds. Birds with long incubation for their body size also take longer to fledge after hatching⁸, whereas those primates with relatively long gestation lengths wean their offspring late⁹.

Differences in patterns of reproductive investment found in the primates are also apparent when comparing orders of mammals. For example, primates have longer gestation lengths and slower postnatal development for their body size than do other mammals: gestation is two to four times longer than in other mammals of equivalent weight, whereas milk production per unit time is far lower^{10,11}. In this respect, humans turn out to be fairly typical primates, having low daily energetic costs of gestation and lactation. Humans are, however, atypical in one important respect: females have large subcutaneous fat reserves¹² and are thus further protec-

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*Reproductive Energetics in Mammals. Zoological Society of London Symposium, 10-11 April 1986. Organized by Andrew Loudon and Paul Racey.

ted from short-term energy crises during pregnancy and lactation.

A.M. Prentice and R.G. Whitehead (Cambridge University) presenting their own and others' work, conclude that the costs of reproduction in humans may be even lower than previously estimated. Important and previously unrecognized factors are a drop in basal metabolic rate over the first trimesters of pregnancy (which can be large enough to mean a net saving over the whole of pregnancy) and, for many women, a further drop in basal metabolic rate during lactation. Perhaps we should not be surprised that, under conditions of acute food shortage, birth weight is decreased by no more than 10 per cent, and that lactation capacity appears to be largely unaffected (except per-

haps in women with primary protein deficiency). Prentice and Whitehead, however, were at pains to point out that although "human pregnancy and lactation are remarkably resilient to the effects of energy restriction", babies born in the Gambia with a "biologically trivial deficit in birthweight of about 5 per cent" have significantly increased mortality².

Energy is a resource that can have a profound influence on reproductive success. Although the importance of energetic limitations have, on occasion, been over-emphasized by biologists, it can be equally unwise to overlook the energetic costs of biological processes. □

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Laser technology

Microfabrication of thin films

from Ian W. Boyd and F. Micheli

THERE is a growing realization that laser-beam processing could provide a powerful and versatile technology for manufacturing thin films and surface structures, and this has remarkable implications for microelectronic, optoelectronic and microwave devices. In recent years this realization has stimulated research interest in laser processing of thin films in almost every major semiconductor industry. The many recent publications and international conferences devoted solely to this topic¹⁻⁴ show that lasers can accurately and controllably perform etching, doping, alloying, surface cleaning, crystallization, oxidation and deposition — indeed, all the processes required to fabricate microelectronic circuits. Even more excitingly, because of the tunability, high intensity and focusability of the radiation emitted by lasers, completely new regimes of processing can now be attained.

Technological interests in this area are abundant. For example, as very large-scale integration (VLSI) devices shrink towards sub-micron dimensions, reductions in processing temperature become necessary to improve resolution and to

minimize defect densities. Lasers offer both a controllable decrease in high-temperature processing times and many alternative low-temperature methods of film and pattern formation. In optical lithography, ultraviolet radiation from excimer lasers is already replacing conventional light sources, thereby maximizing the resolution of the photoresist definition.

Because of the complex nature of energy redistribution within matter, many different routes to laser-initiated reactivity are possible. Often photothermal contributions can be identified in primarily photochemical reactions. There are usually also subtle but important influences on the reaction arising from surface phenomena. Consequently, the area of laser-surface chemistry presents opportunities to observe many new phenomena, but also presents problems in understanding photon-enhanced chemical reactions.

There are two main modes of operation in laser processing, pyrolysis and photolysis, each defining specific bond-breaking mechanisms. In the former, chemical reactions are stimulated by supplying ther-

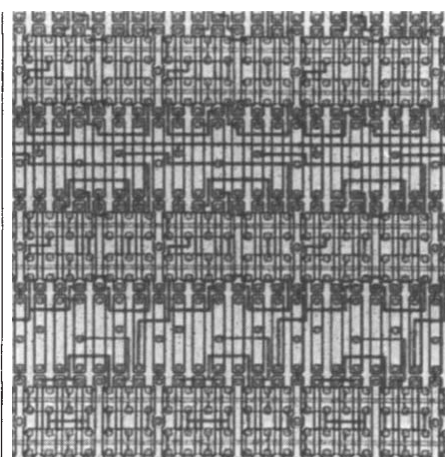


Fig. 2 A MOS gate array of 1,000 gate logic circuits interconnected with laser-deposited poly-silicon in a direct-write mode at Lawrence Livermore National Laboratory (courtesy of Bruce M. McWilliams).

mal energy to reduce the reaction barrier. This is conventionally achieved by furnace heating, but a strongly absorbed laser beam can also induce the temperature necessary for deposition, alloying, diffusion, oxidation or evaporation.

Photolysis involves direct stimulation of a bond through a precise matching of the energy of the individual photons to particular electronic or vibrational levels in the irradiated species. When a sufficient number of photons have been absorbed, selective dissociation occurs, thereby creating reactive species that promote, say, film growth or material removal. In this way many types of gas-solid interactions can be initiated.

Laser etching and removal of oxides, nitrides, metals, silicon and gallium arsenide has led to maskless direct patterning, thereby dispensing with conventional photolithographic methods. The delineated pattern is realized by 'drawing' the focused beam across the film through electronic control of positioning stages. The etching reaction occurs around the area of substrate illuminated by the laser beam. An alternative technique involves direct imaging of the mask pattern on to the film using the intense processing beam (Fig. 1).

The complementary process of thin-film growth (or deposition) has also been intensively studied. In fact, direct writing, or laser pantography, has been used at Lawrence Livermore National Laboratory successfully to produce the first functional *ab initio* multi-layered MOS (metal-oxide-silicon) transistors⁵. Figure 2 shows a poly-silicon interconnect pattern obtained by the Livermore group written by a computer-controlled laser beam⁶. Patterned deposition by mask imaging has not been studied nearly so extensively.

The technology is clearly still at the investigative stage. Whether it will be

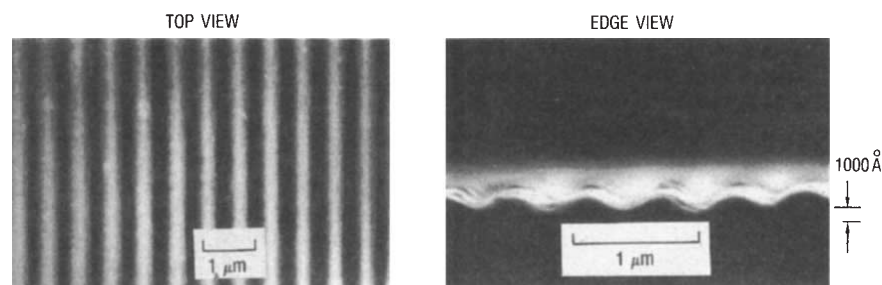


Fig. 1 Pattern-etched molybdenum by projection exposure technique. Conditions: 193-nm laser photolysis of 760-Torr NF_3 , fluence 36 mJ cm^{-2} ; Ronchi pattern projected with Schwarzschild objective (G.L. Loper and M.D. Tabat, Aerospace Corporation, Los Angeles).

accepted by the microelectronics industry, where present production has been fine-tuned and optimized by a deliberate and complex programme over many years of intensive investigation, depends on several criteria. New methods can only be accepted if they can be fitted successfully into the production line with minimal disruption. Many laser-processing methods require major redesign of the existing production lines.

Throughput and reliability are also major considerations. We have calculated that it would take almost one month to draw a metallization pattern on a 6-inch wafer using a half-micron diameter laser beam and a rapid deposition rate of one micron per second. For the imaging techniques to be superior in this application, large-area high-power beams would be necessary. Although several years ago the field was somewhat limited by the availability of reliable high-power ultraviolet lasers, there has recently been dramatic progress in excimer laser technology that make this processing mode more realistic.

The essentially 'one-step' maskless processing capability does present a range of modest applications in VLSI that require only discretionary processing, or corrective 'surgery'. Discrete material removal (by etching, ablation or evaporation) filling-in (by deposition), or rerouting (by direct writing) of particular features is possible on a low-volume basis on newly fabricated or prototype masks, thereby dispensing with design correction and costly manufacture⁷.

Similar operations are also feasible on

low-volume complex circuitry and on customization of chips using elective interconnection of prefabricated gate arrays or programmable logic arrays. In this way, the development of prototype circuits may be speeded up, through interconnection of predesigned cells on 'generic' chips⁸. Additionally, areas of the circuit that are notoriously difficult to fabricate and that exhibit very low yields can be reproduced many times on the same chip and wired in afterwards using localized metal deposition when one particular cell is found to work satisfactorily.

For the full potential of this emerging technology to be realized, there is a need both for more extensive research into the fundamental aspects and for increased effort in developing more useful precursor compounds and even more powerful and reliable ultraviolet laser systems. □

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Immunology

Approaches to tolerance in man

from Leslie Brent

THE title of a recent meeting* suggests that the immunologist's El Dorado — specific unresponsiveness in man — is within our reach. Alas, this proved not yet to be the case. What emerged clearly enough was that it is now possible to manipulate the immune system of experimental animals, from rodents to non-human primates, in various ways so as to achieve functional tolerance of skin, kidney, heart or bone marrow allografts, and that this is almost invariably accompanied by the appearance of suppressor cells.

There are all manner of difficulties in applying these models to man, for some of them are highly complex and others depend on genetic tricks that can be performed only in experimental animals with a short gestation period, such as rodents, but the miniature swine, too, seems to

lend itself to this kind of selective breeding (D. Sachs, National Cancer Institute, Bethesda).

A ray of hope was provided by the technique of total lymphoid irradiation (TLI), pioneered for the treatment of Hodgkin's disease, when followed by bone marrow transplantation. This technique involves the irradiation of the patient's immune system with a series of relatively small doses (150–200 rad) over a period of time while shielding certain parts of the body. It is immensely safer than whole-body irradiation and is now being applied not only to bone marrow and kidney transplantation but also to diseases thought to have an autoimmune origin such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis.

Although the elusive suppressor cell provided a link between many of the very heterogeneous contributions to the conference it is fair to say that, in the context

of autoimmunity, TLI has so far merely provided a reasonably safe form of non-specific, although long-lasting, immunosuppression, with tolerance to the offending and unknown autoantigens still to be demonstrated. One interesting spin-off was the observation (B. Bresnihan, St Vincent's Hospital, Dublin) that TLI given to rheumatoid arthritis patients appears to retard the rate of radiological change in the joints without in the least affecting the level of rheumatoid factor, thus throwing some doubt on the causative role of the immunoglobulin M factor.

The ray of hope alluded to above was provided by TLI in combination with transplantation of bone marrow cells matched for antigens at the major histocompatibility complex (HLA) and depleted of T cells. This was applied in several protocols (with and without chemotherapy and in one group with additional fractionated doses of whole body irradiation) to patients suffering from aplastic anaemia and to a small group of β -thalassaemic children (M. Slavin, Hadassah University Hospital, Jerusalem). The clinical results are extremely encouraging. It is, however, questionable whether the tolerance of the donor cells to host antigens can be attributed to the irradiation rather than to the absence of mature T lymphocytes in the transplant and the acquisition of tolerance by newly generated T cells, as happens in both animal and human bone marrow recipients when other forms of immunosuppression are used. Likewise, good survival of cadaveric renal allograft can be obtained by TLI given together with an initial regimen of low cyclosporin and prednisolone (J.A. Myburgh, University of Witwatersrand Medical School). But whereas Myburgh has incontrovertible evidence that TLI leads to donor-specific tolerance in baboons, this has yet to be demonstrated in patients.

The question of whether TLI is good and safe enough to supplant immunosuppressive drugs in routine kidney transplantation is finely balanced and, until further information is provided, I suspect that most transplant surgeons will continue to opt for drug-induced immunosuppression which, in the best centres, is now yielding very good results although side-effects continue to be a problem.

The mechanism preventing graft-versus-host disease after TLI and allogeneic bone marrow transplantation in rodents was the subject of lively debate. S. Strober (Stanford University) reminded the meeting that natural suppressor cells generated by this strategy have a null phenotype, that they can be cloned and that they appear to be closely similar to the natural suppressor cells that Strober's group and others have identified in the spleens of neonatal mice. A subset of neonatal suppressor cells can be cultured in interleukin-3 (M. Jadus, University of

*Approaches to Human Immune Tolerance Asilomar, California, 9–12 March 1986; organized by S. Strober and R. Hoppe, Stanford University.

South Florida and R. Parkman, Children's Hospital of Los Angeles) and are 'mast cell-like' in that they contain granules that can be shown to cytotoxic.

The nature of the cytotoxicity has yet to be elucidated. M. Slavin described the suppressor cells induced after TLI in rodents as thymus-independent, non-lymphoid, large and immature, and suggested that the effect of TLI is to cause a reversion of the animal's lymphoid system to the neonatal state. Examining the thymus after TLI, Strober found both histological and functional abnormalities in the medulla (failure of cell proliferation after exposure to interleukin-1 and a marked drop in interleukin-2 production when cultured with the lectin concanavalin A), which he explained by the premature departure of cells from the thymus.

There seemed to be some consensus that the suppressor cells in many experimental models are large granular lymphocyte-like cells and K. Singhal (University of Western Ontario) showed that such cells, isolated from normal bone marrow, can regulate *in vitro* immunoglobulin M

responses to several non-transplantation antigens. These cells release a heat-stable, low molecular mass glycolipid that is suppressive *in vitro* if antigen is present from an early stage. The view was expressed that the role of natural suppressor cells is primarily to assist with the functional deletion of self-reactive clones, but they could also have great significance in protecting the mammalian fetus against destruction by maternal responses. The study by B. Roser (Institute of Animal Physiology, Babraham, UK) on neonatally induced tolerance was, however, a salutary reminder that suppressor T lymphocytes, in this case anti-idiotypic cells acting together with another, as yet unidentified, cell can be critically important in transplantation tolerance.

In summary, the conference left no doubt that approaches towards human tolerance are varied; that the main objective remains both valid and desirable; and that we still have a long way to go. □

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Visual information

Do computers need attention?

from Anya Hurlbert and Tomaso Poggio

DESPITE ENORMOUS progress in the past few decades, machine vision is still far from achieving the goal that human vision attains with such speed and reliability — in David Marr's words¹, to "know what is where by looking". Recent results on the physiology and psychophysics of visual attention, one example of which is reported on page 693 of this issue², accentuate the gap between machines and humans, and provide a first step to understanding why it is so large and what machines must learn to overcome it.

Paradoxically, what appear to be the simplest tasks for humans may be the most difficult for machines. Consider, for example, recognizing your mother in a sketch of her sitting in the kitchen. You could immediately and effortlessly locate her face, match it with your memory and pronounce it a good or a bad likeness. If the sketch were upside-down you could easily right it for a proper view. You would probably expend the most painstaking scrutiny in determining just which feature was slightly off, but even so, your final judgement would be quick. In contrast, a computer, using the most sophisticated face-recognition routine, would perform the task slowly and incompletely, because it would not know where to start. Given the location of the two eyes in a sketch cluttered with dark, round blobs, a computer could be programmed to search for the mouth, nose and chin at the appro-

appropriate distances and methodically to match each feature to a virtually identical image in its memory. But failing to find the eyes, the computer could not go on to recognize the face.

Object recognition

The difficulty of the face-recognition problem — and, more generally, object recognition — has called into question one of the main assumptions in the construction of a machine that sees as humans do. The assumption holds that the goal of the first stages in vision is solely to determine 'where' things are, that is, to transform the initial image, an array of intensity values, into a map of the scene which records the distance and orientation of each surface point relative to the viewer (the $2\frac{1}{2} - D$ sketch¹). In machine vision the $2\frac{1}{2} - D$ sketch may serve to guide a mobile robot around an obstacle or to control its manipulations as it picks up a tool. But, like the raw image from which it is computed, the $2\frac{1}{2} - D$ sketch is itself simply a large array of numbers. Although it may contain preliminary information for object recognition by assigning a colour or texture to each surface point, it does not tell 'what' things are. The critical task in object recognition is therefore to find the object or its crucial part within an array of intensity values or distances. Until now, many attempts to elucidate object recognition (reviewed in refs 3,4) have assumed

that the relevant object is already located and isolated in the image.

Unlike machines, humans are adept in spotting the salient features of an object. To understand the mechanisms underlying this ability, psychophysicists have investigated visual attention. Treisman⁵

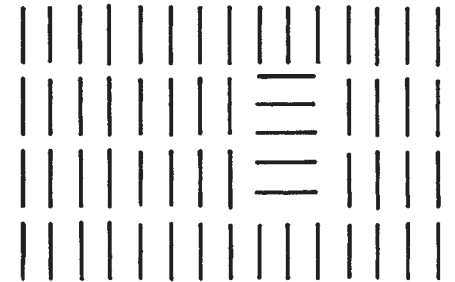


Fig 1. A patch of horizontal line segments pops out in a field of line vertical line segments.

and Julesz⁶ have demonstrated that humans are extremely efficient in detecting a part of an image that differs in a single aspect from its background. For example, a red dot pops out in a field of yellow dots, and the same happens for a horizontal line in a field of vertical lines (Fig.1). The time required to detect the unusual item is independent of the number of other items, implying that the search for it occurs in parallel across the entire field. The human visual system obviously possesses a fast, parallel mechanism which can direct attention to salient chunks of the image. (This is sometimes called 'preattentive'. Here, we consider it as part of the entire attention mechanism whose characteristics probably require more complex descriptions than 'serial' or 'parallel'.) Although the possible computational purposes of this mechanism have not been probed by psychophysical experiments, its potential role in object recognition seems critical. For example, in face recognition the attention mechanism may perform two essential steps: first, to locate 'blobs' which could be eyes; and second, to direct processing towards the blobs to verify that they are eyes and thereby to initiate recognition. The role of attention, therefore, may be not only to spotlight distinctive parts of the image but, more importantly, to segment the image into objects or parts of objects, a crucial first step in determining what things are.

Separable features

An important and still open question is: what are the features or primitives that drive attention? Likely candidates are separable features which, by definition, can be attended to selectively and are processed independently and in parallel. Pop-out and texture-discrimination experiments provide a test for separable features and so far have diagnosed colour, line orientation, line ends (terminators) and possibly crossings among the candidates.

Conjunction experiments test whether two or more separable features can combine to produce a higher-order primitive. For example, when a green T in a field of randomly mixed green Xs and brown Ts is the target, it does not pop out, and the time required to detect it increases linearly with the number of background items. Thus, the detection of a particular conjunction of colour and shape appears to require a search over each item in turn, across the entire field. Conjunction experiments thus reveal another aspect of the attention mechanism, a serial searchlight which appears to operate independently of eye movements and does for feature conjunctions what the parallel mechanism does for features.

Until recently, all conjunctions between known separable features had been shown to require the serial searchlight. The recent results of Nakayama and Silverman⁷ reveal a surprising exception to this pattern. In pop-out experiments using fields of small rectangular patterns displayed on a colour television monitor, the authors demonstrate that binocular disparity and motion individually behave as separable features. The conjunction of motion and colour does not; the search for a pattern of blue upward-moving dots is slow and serial across a field of blue-downward patterns and red-upward patterns. Contrary to this trend, conjunctions of binocular disparity and either colour or motion behave as separable features: they are searched for in parallel. (The authors report that when the field splits into two planes, one in front of the other, the search for a conjunction amounts to a pop-out of the unusual item in one plane. Thus, we suggest that it may be possible, using a different kind of motion stimulus, to create separate planes of coherent motion and thereby induce a parallel search for motion-colour conjunctions.)

Experiments

The psychophysical studies on separable features coincide with the recent emphasis on functional localization in visual neurophysiology and neuroanatomy. It is tempting to draw an explicit connection between biology and psychophysics by equating different visual cortical areas with different feature maps; for example, calling area V4 the colour map, MT the motion map and V1 the orientation map. Psychophysics suggests that in a given feature map there exists at each spatial location a collection of neurones each tuned to a different value of the feature (for example, red, green or blue for colour). Although such an organization has not been demonstrated, evidence for segregation of functionally similar neurones in distinct cortical areas is steadily accumulating. From this point of view, the results of Nakayama and Silverman have inter-

esting implications for neurones and feature maps: they preclude the existence of neurones tuned for both motion and colour; predict the existence of neurones tuned to a particular combination of binocular disparity and motion and of neurones tuned to disparity and colour; and suggest that feature maps are replicated at each of several disparity planes. The prediction of disparity-motion-tuned neurones is supported by the recent report⁸ of similar neurones in cortical area MT.

Other recent work on visual neurophysiology puts the emphasis on a different aspect of attention. Rather than address computational questions such as 'what are the salient features?' and 'how does the attention mechanism work?' or the psychophysical question 'is feature processing parallel or serial?', the new class of physiological experiments on alert animals seeks to demonstrate the ways in which attention can modulate neuronal responses. In the course of such experiments, insights into the neural circuitry

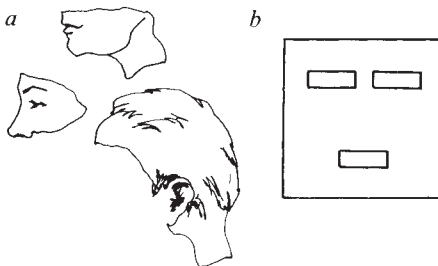


Fig. 2 *a*, each separate set of 'face' features is sufficient to suggest the hypothesis of a face. *b*, It is spatial relation between features and not the features themselves that cue recognition to the face of hypothesis.

and anatomical location of the attention mechanism have emerged. For example, based on studies of attention-mediated modulation in the inferior parietal lobe (area 7), Mountcastle⁹ proposed that neurones there are responsible for directing attention to visual targets.

More recent research demonstrates the effects of attention at other levels in the visual pathway. Moran and Desimone¹⁰ have recently shown that, in the monkey, the response of a neurone in V4 or IT to a preferred stimulus (for example, a red horizontal bar) is dramatically reduced when the animal ignores it and instead attends to an ineffective stimulus (such as a green vertical bar) within the same receptive field (which, for IT neurones, may extend at least 12°). The response of the neurone to the preferred stimulus is unaffected when the attended stimulus is outside its receptive field. Thus, V4 and IT neurones are able to filter out an irrelevant stimulus when it competes with a relevant stimulus within the same receptive field. V1 neurones do not have this property, since the monkey cannot even perform the differential attention task when two stimuli are close enough to fit

within a single receptive field in V1.

The psychophysical experiment in humans reported in this issue by Sagi and Julesz² provides an intriguing complement to the physiological results described above. The authors find that visual attention directed to a random location for an orientation-discrimination task enhances the detection of a test flash presented simultaneously within a certain radius of the target. The area of enhancement, which the authors conjecture to be the area covered by the searchlight of attention, varies from 1.5° at 2° eccentricity to about 3° at 4° eccentricity. Interestingly, these areas are likely to be larger than the average receptive-field sizes in V1.

The above results imply that attention to one region of an image involves both suppression of visual processing in irrelevant domains and enhancement of visual processing in relevant domains. Thus, attention may indeed be responsible for directing a processing focus to specific locations in the initial steps of recognition. Yet although biological research may have found the key to machine vision, it has yet to describe how it opens the lock.

Attention mechanism

Computational results suggest that the attention mechanism is even more complex and powerful than experiments have revealed. Consider again the face-recognition problem. Individual features such as eyes or the curved line of nose and mouth can by themselves lead to the hypothesis of a face (Fig. 2*a*). In contrast (Fig. 2*b*), features alone cannot be the only cue for recognition. The spatial relationship between the two eye tokens and the closed outer contour can also cue the face-recognition process. Ullman¹¹ argues that spatial relations must be computed by a mechanism similar to the serial searchlight of attention.

The unravelling of the full complexity of visual attention will clearly involve computational, psychophysical and physiological research. It will influence not only our understanding of visual perception but also the architecture and the control structure of machine vision systems. □

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Epstein-Barr virus

Infection and tumour induction

from M. A. Epstein

EPSTEIN-BARR (EB) virus, a herpesvirus of man, resembles all other herpesviruses in establishing a persistent carrier state throughout life following initial infection. It has long appeared to infect B lymphocytes exclusively and this specific tropism has been explained by the recent demonstration that the B-lymphocyte C3d receptor CR2 for serum complement also acts as the virus receptor. Infected individuals, however, shed virus into the saliva and the cellular site of viral replication in the epithelium-lined oropharynx has been an enigma. So too has been the oncogenic role of the virus in undifferentiated nasopharyngeal carcinoma, a tumour whose malignant epithelial cells always carry the viral DNA. The finding of virus receptors on epithelial cells¹ and of a viral transforming gene² has now shed the first light on these interrelated problems.

Because only B lymphocytes could be infected experimentally by EB virus it was easy to understand the presence of latent infection readily demonstrable in a percentage of the circulating B cells of infected subjects. For similar reasons, it was assumed that the productive infection responsible for the infectious virions shed into saliva probably arose from infected B cells within the lymphoid tissue of Waldeyer's ring — including tonsils, adenoids, Eustachian cushions and lingual follicles. Hints that epithelial cells might be involved proved difficult to interpret in the absence of a satisfactory mechanism whereby the virus could enter and infect them. But early claims that EB virus replicated in oropharyngeal epithelial cells during primary infection accompanied by infectious mononucleosis were eventually supported and there were also reports that the viral DNA was present in both salivary gland and normal nasopharyngeal epithelial cells. Furthermore, a small proportion of epithelial cells from the human uterine cervix, not usually thought to be associated with EB virus, could apparently be infected after appropriate manipulation *in vitro*.

Striking and unequivocal evidence for EB virus replication in epithelial cells has recently been obtained³ in the context of the immunological disarray which accompanies the acquired immune deficiency syndrome (AIDS). 'Hairy' leukoplakia of the tongue is a newly described lesion of homosexual males suffering from characteristic immunodepression involving absent or poor responses to antigen skin tests and altered, or frankly inverted, ratios of helper to suppressor T lymphocytes. The patients either have AIDS or are likely to

develop it and large numbers of biopsy samples of the tongue lesions have been shown by electron microscopy, specific immunofluorescence tests, and DNA hybridization using EB virus probes in Southern blots, to have EB virus replicating in prickle cells³; a papillomavirus was frequently also present, sometimes even in the same cells.

The crucial importance of specific cytotoxic T cells in maintaining the virus-host balance in the life-long EB virus infection has come to be recognized in the past few years. The balance is delicate, and impairment of T-cell surveillance predisposes to virus-induced pathology. It has been demonstrated repeatedly that transplant recipients on immunosuppressive therapy to prevent graft rejection have depressed numbers of cytotoxic T cells and increased amounts of EB virus shed from the mouth; they also have a heightened incidence of EB virus-associated lymphomas. There is thus a clear parallel with certain aspects of AIDS in which cytotoxic lymphocytes are depleted, unusual EB virus production takes place as seen in the epithelial prickle cells of 'hairy' leukoplakia³, and lympho-

mas carrying EB virus are common. It is of great interest that attacks of falciparum malaria are likewise accompanied by a fall in T-cell numbers and inversion of the ratio of helper to suppressor T cells, since the hyperendemic form of this disease has been recognized for many years as an essential co-factor in the induction of virus-related endemic Burkitt's lymphoma.

Interactions between EB virus and epithelial cells are no longer mysterious. Using two monoclonal antibodies against different epitopes of the complement C3d CR2 EB virus receptor, Young and colleagues¹ show that the receptor molecules are present on the cells of oral and nasopharyngeal squamous epithelia and, in view of the hairy leukoplakia findings, it may be significant that on the tongue the receptors appear to be restricted to the prickle cell layers. All the evidence now supports the view that permissive productive replication of EB virus takes place in oral and pharyngeal epithelial cells, which fits well with the ten-year-old observation that malignant epithelial cells of nasopharyngeal carcinoma, which carry the virus genome, can be readily induced to produce infectious virus both in tissue culture and in nude mice.

If entry of EB virus into epithelial cells can now at last be understood, what of its role in changing them into cancer cells? As far as nasopharyngeal carcinoma is concerned, nothing has hitherto been clear,

100 years ago

Power in Laboratories

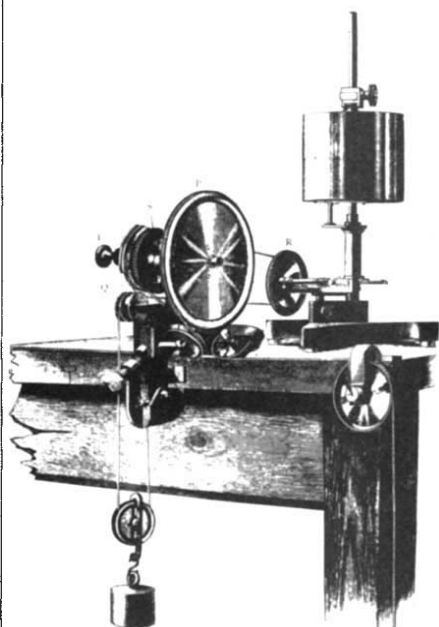
IN connection with the admirable devices for the distribution of driving-power in laboratories, illustrated in *Nature*, vol. xxxiii. p. 248 (shown opposite), the description of a novel and very effective form of water-engine, with which I have been experimenting for several months, will be of interest.

One of these motors is set up in the cellar of our science hall, where it is supplied with aqueduct-pressure of sixty pounds to the square inch, and the power is transmitted from it by means of rubber belting led over

crossbar which engages the crank of the main shaft. Thus the ring, in turning the shaft, has the vibratory motion of an eccentric, and returns the opposite pistons to the bases of the cylinders, at the same exhausting the water through the interior of the rotary valve.



"idle pulleys" to the upper stories of the building, where a small engine-lathe and dynamo are driven. A word will suffice to explain the very simple construction of the motor-system of radial cylinders, with their bases at the centre of the motor, through which runs the driving-shaft. The pistons in these cylinders are single-acting, and the water is admitted to them in succession by the rotary valve which forms part of the main shaft. The pistons, thus, in pressing outward, exert their force against a strong ring, to which is bolted a



From *Nature* 34, 121; 10 June 1886.

although racial, genetic and environmental co-factors are necessary. On the other hand, enough has been learned about EB virus in relation to endemic Burkitt's lymphoma for a persuasive theory to have emerged⁵.

Thus, it has been suggested that latently infected B cells undergo unusually abundant virus-driven replication, no doubt because of impaired T-cell control resulting from recurrent attacks of falciparum malaria, such that one or other of three specific chromosomal translocations occurs, each being a cause of *c-myc* oncogene activation. Serious doubts have been raised, however, as to whether cellular oncogenes are relevant at all to the induction of cancer⁶, and certainly *c-myc* alone cannot render cells cancerous⁷.

New information on a key EB virus gene promises to reconcile these conflicting views. The gene for the virus determined membrane protein of latently infected cells has been identified and cloned, and after transfection into quite alien cells expression of the product has been shown to confer tumorigenicity⁸.

This transforming gene explains the ability of EB virus to cause malignant tumours rapidly and directly in susceptible animals⁸, and clearly plays a major role in the chain of events leading to the development of endemic Burkitt's lymphoma, whether with or without interaction with an activated *c-myc* oncogene. Discovery of this gene also provides a first and important indication as to how EB virus might be involved in causing nasopharyngeal carcinoma and, indeed, the other epithelial tumours in which preliminary investigations have identified the viral genome. □

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Plant ecology

Pesticides and pollination

from Peter D. Moore

INTERDEPENDENCE in nature is often regarded as a source of strength and stability, but it can also become a point of weakness when one member of a complex chain of interactions is affected by catastrophe. In the case of insects involved in flower pollination, for example, there are evident evolutionary advantages in specialization that involve interactions between individual plant species and specific vectors, but such relationships may lead to problems if the vector population becomes depleted as a result of catastrophe. And catastrophe in insect populations is a far from unlikely event in these days of broad-spectrum pesticides. This problem is the subject of a recent study on the consequences to the reproductive success of plants in a New Brunswick forest of pesticide spraying (Thomson, J.D., Plowright, R.C. & Thaler, G.R. *Can. J. Bot.* **63**, 2056; 1985).

In the coniferous forests of New Brunswick, the spruce budworm (*Choristoneura fumiferana*), a defoliation agent of balsam fir (*Abies balsamea*), is an economically significant pest. Various pesticides have been sprayed onto the forests in the past 25 years in efforts to control the budworm, but concern has been expressed about the possible effects of spraying on other, non-harmful insect species and even economically beneficial species, such as the bees involved in pollination, and therefore fruit production, in the blueberries (*Vaccinium*

spp.) that grow in the same area. P.G. Kevan (*Biol. Conserv.* **7**, 301; 1975), studying the pesticide fenitrothion, provided evidence that such effects can indeed be harmful.

Thomson and his co-workers investigated the effects of spraying Matacil, an anti-budworm agent which, because it is less toxic to bees, has replaced fenitrothion. When they tested the effects of Matacil on the mortality of a range of wild insect species, Thomson *et al.* found that the most pronounced effects occurred among the smaller bees (Andrenidae, Halictidae and Anthophoridae) and one fly family, the Syrphidae. These families are important pollinators of some of the understorey plants such as the Canada mayflower (*Maianthemum canadense*) and the red-osier dogwood (*Cornus stolonifera*). But the question to be answered is whether the spraying influences the populations of these insects to a degree that affects the breeding success of these plants.

By studying fruit sets in *M. canadense*, *C. stolonifera* and the creeping dogwood (*C. canadensis*) both inside the sprayed area and in control plots outside the influence of the spray, Thomson *et al.* obtained an estimate of fecundity, expressed as a percentage of the total flowers on an individual achieving fruit set. In *M. canadense* and *C. stolonifera* they found a significant reduction in fecundity within the sprayed

area (means of 4.6 and 8.8 per cent, respectively) compared with the control sites (9.7 and 24.6 per cent). But in *C. canadensis* there was no significant difference (spray mean of 13.1 per cent and control mean of 11.6 per cent). The discrepancy between the two *Cornus* species could be caused by the fact that *C. canadensis* is also pollinated by larger bumblebees (*Bombus* sp.) which are not affected by Matacil spraying. These results add weight to the argument that spraying where the pollinator is sensitive decreases fecundity.

The question of whether the decrease in plant fecundity actually influences its overall population stability still remains unanswered. Much depends on the degree to which the plant requires the seed as a survival and/or dispersal mechanism. If vegetative growth and dispersal are available as alternative strategies for a species, then the decrease may be of little consequence. Although there are exceptions, such as the pin cherry (*Prunus pensylvanica*) (Marks, P.L. *Ecol. Monogr.* **44**, 73; 1974), most woodland understorey species are long-lived perennials with little dependence on their seed set.

Where the effects I have described here are likely to have the most profound impact is in plants which have a limited geographical range, are out-breeding, perhaps dioecious, short-lived, have no capacity for vegetative propagation and are specific in their pollination vectors. A plant which answers to most of the elements in this description is the hop tree (*Ptelea trifoliata*), a rare plant that colonizes shorelines on the northern side of Lake Erie in Canada. J.D. Ambrose, P.G. Kevan and R.M. Gadawski (*Can. J. Bot.* **63**, 1978; 1985) have studied the reproductive biology of this species and conclude that it is dioecious, is entirely dependent on wind-dispersed seeds for its spread and exhibits a rapid population turnover. Its breeding would therefore seem particularly susceptible to any catastrophic disruption such as that in the New Brunswick forests. But there is one factor in its favour — a considerable diversity of insect vectors in its pollination system. Ambrose *et al.* found that the flowers were visited by Hemiptera, Coleoptera, Diptera and Hymenoptera, totalling at least 102 species from 37 families. In this particular respect, what seems to be a vulnerable plant has a survival strategy and the ability to withstand the almost inevitable impact of pesticides on its pollination vectors. Perhaps we should give special attention to the conservation of those plant species which lack this key factor in their defences against human environmental disruption. □

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Iodine-131 in human thyroids in Britain following Chernobyl

SIR—On 2 May 1986 raised atmospheric levels of radioactive material were detected in the southern United Kingdom, apparently due to the Chernobyl reactor accident. Some activity persisted subsequently, but at a level considerably lower than the initial peak¹. One of the most serious consequences believed to result from the accidental release of fission products from irradiated fuel is irradiation of the thyroid gland by ¹³¹I (ref. 2). Thus, particularly because a substantial component of the radioactivity detected was identified as ¹³¹I, it seemed desirable to carry out some direct measurements of ¹³¹I in human thyroids. Between 10 May and 22 May we carried out a series of measurements on the thyroids of adults and children resident in the southern United Kingdom (predominantly South London) during the period of exposure.

Measurements were taken in a chalk-shielded room with a low radiation background, using a high purity germanium detector, collimated to be sensitive mainly to the thyroid region and with an overall counting efficiency for ¹³¹I of 0.27%. Of necessity, subjects were chosen on the basis of availability and willingness and consequently consisted predominantly of departmental staff and students, together with five children of staff. None of the individuals for whom data are reported here had been occupationally exposed to ¹³¹I in the recent past. However, to check for the possibility of inadvertent contamination in the hospital environment, we carried out identical measurements on three staff employed full time in our clinical radioisotope dispensary and found

their ¹³¹I levels to be within the range observed for the test group. Measurements were commenced over the weekend of 10–11 May and continued thereafter. Measured values of activity in the test group of subjects, and corresponding dose-rates calculated using data of International Commission on Radiological Protection^{3–5}, are presented in Table 1. From these data it seems reasonable to conclude that the peak in thyroid activity for most subjects occurred in the period between 14 and 19 May.

To estimate, from the measured activities, the time integral of dose commitments to the thyroid that may be expected to result from these exposures, we have made use of the data from a parallel set of measurements that we have carried out — commencing on 8 May and using similar measurement technique — on ¹³¹I concentrations in milk delivered by a local dairy. The results of these measurements are presented in Table 2 and appear to be consistent with those observed at Chilton (some 100 km north-west of Sutton) which peaked on 6 May at 54 Bq l⁻¹ and were made on farm-fresh milk¹. If it is assumed that approximately 0.5 l of milk, ingested in the 24 hours following each day's delivery, was the main source of ¹³¹I in the subject group^{1,6}, then the data are consistent with the ICRP model in which 30% of ingested ¹³¹I is deposited in the thyroid, where it remains with half-life of 7.6 days^{4,5}. On this basis the appreciable increases in thyroid activity (10–65%) during the week following 10 May further suggest that the peak activity in milk delivered in Sutton did not arrive until some 2 days following that observed for the Chilton farm, coinciding with the start of our own measurements on 8 May.

Accepting these assumptions, and assuming also that dietary activity hence-

Table 2 Iodine-131 activity measured in milk from a Sutton dairy

Date of delivery	Activity (Bq l ⁻¹)
8 May	41 ± 5
10 May	34 ± 5
12 May	21 ± 5
13 May	13 ± 4
14 May	11 ± 4
15 May	9 ± 4
16 May	8 ± 4
17 May	8 ± 4
19 May	7 ± 3
20 May	5 ± 3

forth decays with the half-life of ¹³¹I, it is possible to estimate the time integral of thyroid activity resulting from the incident. Such a calculation, consistent both with our limited measured data on thyroids and also with similar measurements following the Windscale reactor accident in 1957⁷ (made later in the time variation in activity), points to a total dose commitment from the incident equivalent to that from the peak dose rate, in each individual, acting over a period of about 26 days. Values derived in this manner (using the highest observed dose rate) are listed in the final column of Table 1. These values can be compared with a figure of approximately 1,000 μSv for the population mean annual dose to the thyroid that arises from natural sources⁸. These values are close to the estimates of ¹³¹I dose to thyroid that resulted from the intensive series of nuclear weapons tests in 1961⁹.

Prior to the Chernobyl incident, one of the world's most serious reactor accidents appears to have been that at Windscale (subsequently renamed Sellafield) in 1957. On that occasion, ¹³¹I measurements were also made for the population centred

Table 1 Iodine-131 radioactivity measured in the neck region (Bq)

Subject	Age(sex)	Measurement date (May 1986)												Assumed mass of thyroid ¹ (g)	Highest observed dose (μSv d ⁻¹)	Estimated dose commit- ment (μSv)
		10	11	12	13	14	15	16	17	18	19	20	21			
Adults																
A1	60(M)		8±4					10±4						20	1.4	40
A2	56(M)	8±4						13±4			7±4		6±3	20	1.8	50
A3	33(M)	21±5					30±5				33±6			26±4	20	130
A4	36(M)	17±5												20	2.4	60
A5	35(F)												8±3	20	1.1	30
A6	35(M)		27±6				30±5					23±4		20	4.1	110
A7	34(M)		17±5						23±4					20	3.1	80
A8	26(F)	15±5				20±4					13±4		20±5	15±4	20	70
Children																
C1	12(M)		8±4											10.2	2.1	50
C2	6(F)	6±5									2±2			4.9	3.3	90
C3	4(M)												8±3	2.5	8.8	230
C4	4(M)												13±4	2.6	14.0	360
C5	2.5(F)		11±6						16±5					2.3	19.1	500

on Sutton (including one of the present subjects) and showed peak levels of ^{131}I thyroid activity at that time ranging up to 300 Bq in adults⁷. The present values are clearly an order of magnitude lower.

It may also be of interest to note that our measurements (made using a simpler, screening procedure) on five UK citizens on their return from residence in Warsaw during the week 28 April–4 May showed levels of ^{131}I in the thyroid (at 4 May) ranging from 40 to 150 Bq. Measurements made on the same date on two individuals returning from residence in Kiev showed thyroid ^{131}I levels of 1,100 and 1,700 Bq. In contrast, the levels in 11 individuals returning from Leningrad and Moscow and two from parts of West Germany were all below the 40-Bq detection limit in our screening measurement.

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Do mammals need P elements?

SIR—In a recent *News and Views* piece, Andy Flavell describes a way by which the *Drosophila* P element has been engineered to eliminate the germ-cell specificity of synthesis of the P transposase and allow transposition of P elements in somatic cells¹. As he says, these modified elements may well direct synthesis of transposase in mammalian cells, and cause transposition of P elements around mammalian genomes. Would this then give vertebrate molecular biologists all the advantages given by P elements to *Drosophila* geneticists? The answer, I fear, is no.

The P elements have proved to be yet another powerful addition to the already well-equipped armoury used in solving the problems of developmental biology of *Drosophila*. They provide tools for both isolating and analysing genes. It is the prospect of easy transposon tagging of mutations and a high efficiency of production of transgenic animals that has

tempted mammalian developmental biologists and geneticists (myself included) to yearn for a P-like element in mammals. However, sober reflection suggests that even if this were come about, we would be only slightly, if at all, better off.

None can doubt the enormous potential of *Drosophila* biology for adding to our understanding of the genetic control of development of a complex animal. Much of this potential stems from the wealth of mutations which affect development in surprisingly precise ways. With this fount providing the raw material those who work on *Drosophila* can count their further blessings; the use of polytene chromosomes to correlate genetic with physical maps, the small genome which facilitates cloning and chromosome walking and the tricks of the P elements. It is, however, the genetics of *Drosophila* upon which all else hangs, and this genetics in turn has become so well-understood because of two key factors: the small size of the animal and its rapid generation time which permit the analysis of many thousands of individuals and crosses.

Amongst the vertebrates only mice have a comprehensive and manipulatable genetics and are small enough to enable large numbers to be maintained. But the two main benefits brought to *Drosophila* by P elements, transposon-tagging and gene integration, already exist in mouse molecular biology. The rate-limiting steps in the advance of these experiments lie elsewhere, in the handling and analysis of large numbers of animals.

Retroviruses have been used as transposon tags for isolating mouse genes for a number of years. Natural infection of the germ-line has produced at least one coat-colour mutation, and allowed DNA from the region to be isolated². Infection of embryos *in vitro* has been used as a method of generating insertional mutants, and their subsequent isolation³. Additionally, a spin-off from producing transgenic mice, which have insertions of foreign DNA, has also been the isolation of mutations caused by the insertion event^{4,5}. These methods are all technically difficult and slow. More efficient *in vitro* viral infection of embryos would speed things up and methods have been described which yield about 10% of offspring with new viral inserts in the germ line⁶. *In vivo*, appropriate crosses between Swr and RF mice — in a sense analogous to a dysgenic cross of *Drosophila* — result in 0.35 to 0.67 new retroviral inserts per animal⁷, a rate that begins to approach that of P elements.

Testing carefully for new recessive mutations caused in the mouse by DNA insertion entails a great deal of cross-breeding, DNA preparation and Southern blotting, but there is a good chance of identifying embryonic lethals⁸ (rather few of which are likely to be 'developmental'

mutations). Setting up sib-crosses without DNA analysis and simply waiting for a new recessive mutation to reveal itself would reduce the work required. Of course this would only allow identification of viable mutations, and is extremely inefficient as even under the best circumstances the vast majority of crosses would be between animals not carrying any inserts. If one were to look for a *specific* mutation caused by an insertion the logistics become daunting to say the least. If one were to take all the offspring of Swr/RF crosses 'blind', and backcross them to a tester stock, assuming a target size for mutation of 20 kilobases and a genome size 100,000 times that, a rate of new insertion of 50% overall would produce the desired mutant once in 200,000 mice. Using a multiple tester stock carrying ten or twenty interesting genes would hardly reduce the scale of the task to one which the average worker would tackle. Few mouse facilities house more than twenty thousand animals in total. Even if only ten thousand crosses were needed (routine in a *Drosophila* lab), this would occupy a good sized room and several animal technicians for a couple of years. We would have to be confident indeed that the gene being tagged was truly interesting to justify such efforts.

Introduction of foreign genes into the mouse genome by microinjection of the embryo is now a widely used method of analysis of gene function. Despite early setbacks it now seems that most intact human or mouse genes will function after microinjection⁹. The efficiency at which injected embryos integrate the foreign DNA varies between 5 and 30%, which is about the same as the efficiency of P-mediated integration after injection. There is, at the moment, no marker which allows identification of transgenic animals, other than DNA analysis. The rate-limiting step, however, is the number of embryos which can be readily handled. It will always be a very skilful task to handle, inject, culture and replace the embryos into foster mothers.

Integration mediated by the P transposase almost always results in only a single integrant. Transgenic mice very frequently have multiple, tandem inserts, although there seems to be no correlation between insert number and expression of the genes⁸. Retroviral vectors can be employed to produce transgenic mice with only single inserts⁶ but there are size restrictions on the DNA that can be introduced in this way. It is here that appropriately modified P elements may be most useful, allowing the easy generation of single-insert transgenics.

All this said, even if transposing P elements onto the mouse genome and having them jump around there will not push mouse biology into the same league as *Drosophila* biology, it would be worth-

while and informative. Moreover, it sounds like fun!

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Initiating boiling with ice

SIR—When an observation in the physical world appears to be paradoxical, running counter to one's intuition, experience and learning, it usually provides an opportunity to achieve some valuable scientific insight. Such a case began with the observation by one of us (R.L.D.) that a large spoon that had been kept in the freezer would initiate boiling in water that had been heated in a cup in a microwave oven, which the other one of us (R.E.A.) suggested might occur because crystals of ice initiate boiling in overheated water.

Water is said to be overheated or 'superheated' when its boiling temperature for a given pressure is surpassed without transformation to the vapour phase. This can be brought about by heating a drop of water as it rises in a heated column of oil. Using this technique, water can be heated to as high as 279.5°C at atmospheric pressure before explosive boiling occurs¹. This extreme superheating occurs because there is no free vapour/liquid interface, the water being immersed in oil. Even if there is a free interface, significant superheating is possible² because the internal temperatures can rise above the boiling temperature which occurs at the interface where vaporization occurs. (The true definition of boiling relates to vaporization at a flat liquid/vapour interface, and, therefore, is not defined by the onset of vapour bubbles.) 'Bumping' occurs when clean water is heated and boils unevenly but can be avoided by the presence of a boiling 'chip' or a piece of Teflon, which contains many gas-entrapping crevices.

We found that the temperature that could be achieved by tap water in a beaker heated on an oven-top burner was $100.75 \pm 0.25^\circ\text{C}$, close to the boiling temperature. But when tap water was heated in a microwave oven, the temperature measured by a thermocouple was briefly in excess of 110°C and there were more sustained periods of 105 – 106°C . To ensure

that our thermocouple did not trigger boiling, we put it in a glass well that contained cooking oil. The tip of the glass well was positioned in the centre of the tap water held in a Pyrex beaker. The leads to our thermocouple were not attached to the voltmeter while the microwave was running, because the radiation would cause spurious readings. Our chromel/alumel thermocouple was calibrated with a crushed ice-water bath, and checked at the boiling temperature using water brought to a rolling boil (using a large chunk of Teflon).

Several kitchen objects were used to check the hypothesis that ice crystals nucleate boiling in ordinary tap water. When a stainless-steel or silver serving spoon was used, it would only nucleate boiling when cooled in a freezer (where some ice crystals could form). Glass, a screwdriver, and a plastic utensil behaved in the same way. If small boiling chips were added to the water before it was heated, we could not produce enough superheating, and inserted objects did not stimulate boiling.

Ice cubes themselves triggered a 'rolling' boil if the temperature rose above about 102°C . At a superheat of about 10°C above the boiling temperature, ice could nucleate boiling for 3–4 s. The time of boiling roughly scales to the number of degrees of superheat. Ice made with degassed water behaved in the same way as ice made with tap water saturated with gas.

It is quite clear that the mechanisms of the phenomena we observed are as follows: the microwave oven heats the water directly and the container indirectly, the opposite of what happens when heating by conduction with a burner. Therefore, the internal temperature can rise above the boiling temperature at positions away from the container's surface which tended to provide sites for boiling. Evaporation at the free surface does not occur rapidly enough to cool the bulk of the sample. When an ice cube is introduced, its creviced crystalline structures, which can hold gas or water vapour, stimulate film boiling on local parts of the surface of the ice cube. The vapour film acts as a partial thermal insulation, slowing down the heat transfer between the ice cube (at about -15°C) and the water (at 110°C). When the water temperature immediately around the ice cube drops below 102°C , or so, the observed boiling ceases.

When our experiments are repeated with water in a plastic cup, a much smaller degree of superheat is achieved, primarily because the water wets plastic much more poorly than it does Pyrex glass, and therefore boiling on the surfaces of plastic is much more active, cooling the interior and preventing significant superheating.

One word of caution. We used extremely fine, insulated thermocouple wire, which allowed us to close the microwave

oven door and not risk radiation leakage. Similar precautions should be taken by those who desire to repeat our experiments.

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In defence of bacterial phylogeny

SIR—In recent correspondence, Woese *et al.*¹ say that our differences on *Archaeobacteria* eocytes and photocytes are semantic. Our differences are fundamental and are based on two contradictory evolutionary trees. If one tree is correct the other is not. These trees are our combined photocyte/eocyte tree^{2,3} and the tree of Woese¹. Each relates all extant organisms.

The most widely used and accepted method for selecting a tree is parsimony analysis⁴. The four prokaryotic groups, halobacteria, eubacteria, methanogens and eocytes, can be topologically connected (by unrooted trees) in only three different ways. These correspond to our photocyte tree, to Woese's tree and to a third alternative, shown in Fig. 1. According to parsimony, a tree relating four taxa is supported if a given molecular property is found in two taxa while an altered version of the property is found in both of the other two. Thus "two-and-two" properties are valid. However, if a property is found only in one of the four groups, then it does not support any tree. Hence "three-and-one" properties are invalid.

All of the photocyte molecular properties listed in Table 1 below support the photocyte tree. All are two-and-two and hence are phylogenetically valid properties by the parsimony criteria. The list includes basic cellular properties such as membrane related components [tetraether lipids⁵ and fatty acid esters⁶], biochemical pathways [carotenoid synthesis⁷ and arginine deiminase pathways⁸] and bioenergetic components [C_{40} and C_{50} carotenoids⁹ and 2Fe-2S ferredoxin¹⁰] in addition to ribosomal RNA sequences and ribosome structure. Contrary to the implication of Woese *et al.*¹, the eocytic 50S lobes found in ribosomes of eocytes and systematically distributed within the methanogens^{2,11,12} are also two-and-two. All molecular characters are the same for halobacteria and eubacteria and for methanogens and eocytes, hence they all support the photocyte tree. Many are

deeply integrated into the molecular fabric of these cells. These are precisely the features expected to be good evolutionary markers.

No properties support the Woese tree, including the "founding" ones upon which both the tree and classification were proposed, "membrane, cell walls, rRNAs and tRNAs". These "founding" properties are listed in Table 1. For example, 16S rRNA oligonucleotide catalogues indicate that the eubacterial pattern is different, but they do not identify which group the eubacteria are closest to. All of their characters are three-and-one and cannot support any tree. When judged by standard evolutionary procedures and tests, the same "founding" properties which prompted Woese's taxonomic proposal, fail to support his tree.

Ribosomal RNA sequences, as rapidly evolving molecular characters, are subject to artefactual analysis by parsimony. No matter what the correct tree, parsimony and other treeing algorithms can consistently predict a tree which, like Woese's places fast-clock groups (that is, eubacteria and eukaryotes) together¹³. When we analyse the rRNA sequence data using the method of phylogenetic invariants, which is insensitive to this artefact and independent of evolutionary rates, we find support for our eocyte/photocyte trees is significant at greater than the 99% level^{14,15}.

Woese claims that his proposed classification could be used with any tree. A classification that fits all trees is no classification at all. Consider the arginine deiminase pathway found in the photocytes (eubacteria and halobacteria). We

Table 1 Prokaryotic molecular properties

	<u>Eu-</u> <u>bac-</u> <u>teria</u>	<u>Halo-</u> <u>bac-</u> <u>teria</u>	<u>Me-</u> <u>than-</u> <u>ogens</u>	<u>Eo-</u> <u>cyt-</u> <u>es</u>
VALID BY PARSIMONY (Photocyte)				
Arginine de- iminase pathway	+	+	-	-
Tetraether lipid	-	-	+	+
2Fe-2S ferredoxin	+	+	-	-
C40 carotenoids	+	+	-	-
Fatty acid esters	+	+	-	-
C50 carotenoids	+	+	-	-
Similar carote- noid synthesis pathways	+	+	-	-
Ribosomal 50S eocytic lobes	-	-	+	+
rRNA sequences	SEE DISCUSSION			
IRRELEVANT BY PARSIMONY (Woese's characters)				
Ester lipids	+	-	-	-
Eubacterial cell walls	+	-	-	-
Eubacterial oligonucleotide catalogs	+	-	-	-
F-met initiator tRNA	+	-	-	-

interpret it as having evolved once in the photocytes. Woese's proposed classification, making it a property occurring in two "kingdoms," misdirected others to propose either (1) that it was invented twice, or (2) that it was laterally transferred between "kingdoms". An incorrect classification has great negative power to obscure and misdirect.

Science and not semantics, then, is the basis of our claims. We see no support for Woese's tree and taxonomic proposal from any molecular properties, whereas deep and fundamental properties support our eocyte and photocyte trees and classification. Viewed through the photocyte tree, membranes, biochemical pathways, and components of biochemical energetics are all natural and logical consequences of evolutionary history. Well integrated molecular biological and metabolic properties support the photocyte and eocyte trees and classifications. In the end, evolutionary connections, and not semantic ones, between organisms give theories true organizational and predictive value.

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Testis size and dizygotic twins

SIR—A recent article in *News and Views*¹ discussed R.V. Short's observations² on human testis size and dizygotic twin frequency in Asian, Indian, Caucasian and African ethnic groups.

Large testes were the first clinical feature to be associated with fragile X chromosome-linked mental retardation^{3,4}. A mean testicular volume of 48 ml (range 15–127 ml, modal volume 35–40 ml) was observed⁵ for adult white fragile X males in whom testicular volume was not a factor in ascertainment. The average testicular volume in unaffected Caucasian males is 20 ml. In an ascertainment of fragile X-affected kindred in Hawaii⁶, it was noted that the mean testicular volume (22 ml) of pure Japanese males was less than that (39.5 ml) of pure Caucasian males. This reflected a previously observed difference between unaffected males of these ethnic origins.

A recent Belgian study⁷ of 144 obligate female carriers of the fragile X led to the conclusion that such carriers have a high fertility, especially in those who are mentally subnormal (30%). An increase in the incidence of twinning was observed in the 642 progeny of 134 obligate carriers. Of 18 pairs of twins, 12 were dizygotic, and, in the remaining 6, the zygosity could not be determined. This incidence (1 in 35 births) is three- to four-fold that of the expected incidence of twinning in Caucasians (1 in 80 to 1 in 140 births)⁸. This specialist fragile X-bearing population therefore would appear to express parallel variations to those already observed by your contributors.

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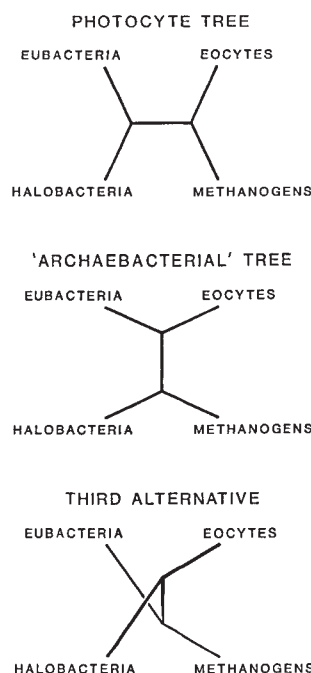


Fig. 1 All alternatives for linking the four prokaryotic evolutionary groups are represented in these three unrooted trees.

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Unnatural science?

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Profiles of Social Research: The Scientific Study of Human Interactions.

By Morton Hunt. *Russell Sage Foundation, New York:1986. Pp.324.*

Distributed by Basic Books, hbk \$17.50, pbk \$8.95.

LONGSTANDING readers of this journal on both sides of the Atlantic may raise their eyebrows at the very title of the book here under review. Natural scientists often deny the legitimacy of using the word "science" in conjunction with social research, a view shared by some people in high places. But surely nobody can doubt that the social world is as much part of nature as the world studied by natural scientists, equally there to be observed, understood, measured, occasionally manipulated in experimental fashion, conceptualized and perhaps even explained.

The denial of scientific status for social research cannot rest on these generally accepted criteria for science. Indeed it does not. The verbal quibble is a substitute for an appreciation of some genuine differences between the natural and the social sciences beyond the unquestioned difference in achievements. There is a distinction between the phenomena in the social and the physical worlds. The regularities social scientists discover are subject to change. Those in the natural sciences are not; they are assumed to hold for all times and through the entire Universe. This has implications for the development of theories on both sides of the fence. In the natural sciences, a new theory should encompass the regularities a previous one has explained. No such demand can be met in the social sciences, because the regularities themselves may have altered. So, while there is no shortage of theories in the social sciences, there are no cumulative explanations, at least not so far. Social scientists must deal with their complex subject matter in an extended "Now", the duration of which is unknown.

The relatively low public prestige of the social sciences is, however, less grounded in this feature of the subject and more in another ineluctable fact: while no lay person can pronounce on the unification of the four forces, not even on a single particle, everybody has daily experience of the social world and holds implicit or explicit theories about it. Depending on such personal views, one and the same social scientific formulation is regarded by some as trivial common sense, by others as false.

Morton Hunt's competent and lively description of several social research projects, their methods, achievements, difficulties and public reception, addresses these issues not directly but by example.

The volume was commissioned by the Russell Sage Foundation of New York, in celebration of its 75-years-long support for the social sciences. It deals understandably but regrettably almost exclusively with American research, but social scientists elsewhere will have no difficulty in recognizing their subject, apart from

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Boston, September 1974 — racial hatred erupts as whites protest against the court-ordered integration of public schools through the bussing of students to different parts of the city.

the incomparably greater funding available in the United States. The book is based on published research and interviews on aspects of the research process that are not normally included in the written record. Hunt conveys above all something that should appeal to natural scientists: the intellectual quality and commitment to high standards that outstanding social scientists invest in their efforts.

Part I gives an illustrated overview of the standard methods of social research, paying due attention to their assets and shortcomings as well as to the ethical problems that inevitably arise when people are the subject of study. Neither advocacy nor critique, this is a fine presen-

tation of the state of the art in social research.

Part II contains detailed descriptions of five major projects, including their internal histories, costs, methods, results and, in one dramatic case, the wide-reaching public consequences that followed publication. This was James Coleman's famous — according to some, notorious — large-scale survey of school segregation and its effects. Coleman's study was commissioned by the government in 1965, with a budget of \$1.5 million, and Hunt describes it by skilfully interweaving the research issues with an account of the social and legal context, an intellectual biography of Coleman and the views of various social scientists on the question of whether social research should remain "pure" by choosing its own topics, or try to answer questions raised by policy-makers. After describing the conceptual and practical problems of conducting a survey of more than 600,000 children in 4,000 schools, Hunt presents the computer-produced results. They shocked the civil rights movement, which had confidently expected they would show that more money spent on desegregated schools would reduce, if not eliminate, the achievement differential between black and white children. Coleman's conclusions were different. For example, he found that parental economic status had more to do with achievement than race or quality of school. Up-roar followed: simplified press accounts, congressional hearings, attack and defence in popular journals. At that time the controversial bussing policy was introduced — and failed. White parents took their children from the integrated city schools and moved out into the suburbs, or put them through private education. And Coleman, re-analysing his data after several years, acknowledged publicly that the original data already contained evidence for "white flight" as a result of desegregation.

It is to Hunt's great credit that he presents this confusing story without minimizing the intellectual and political passions it aroused, nor the mistakes made on all sides, and yet shows convincingly the potential power of social research to illuminate controversial issues. One question, however, he leaves unasked: is it in the best interest of social scientists or policy-makers to demand quick answers to immediate problems?

Case histories of two further government-initiated studies allow Hunt to enlarge on the assets and vicissitudes of social research. One was the decade-long, real-world experiment on negative income-tax, better described as income maintenance for the very poor. Economists, statisticians, sociologists and social psychologists cooperated in this complex project. Hunt uses it as a basis for a sophis-

Popperfoto

ticated exposition of the research process, its relation to theory and, in this case where time was not pressing, for the demonstration that the amount of thought and expertise invested in such research has its intellectual pay-off and its social uses. The other government-initiated study, the Survey of Income and Program Participation (in social welfare programmes), gives Hunt occasion to trace the history of survey research and indicate what is considered to be current best practice.

A fourth case takes the example of a social psychological laboratory experiment with undergraduates to illustrate the great power of this method, which dominates American social psychology, and its inherent problems: Do the results apply to

the world outside the laboratory? Is the desire for experimental precision responsible for all too many elegant trivialities in the literature? A final case exemplifies the methods of longitudinal panel research through analysis of some studies, designed to continue for 20 years, of aging in an originally healthy middle-aged sample.

Altogether this is an engagingly and clearly written book. It presents social science as it is, warts and all. Social scientists could well use it as a textbook. Natural scientists may gain from it insight into the intellectual challenges social scientists face, and respect for their efforts in meeting them. □

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Inflamed appeal

Gerry Higgs & Brendan Whittle

The Inflammatory Reaction. By Henry Z. Movat. Elsevier:1985. Pp.365. Dfl.260.

THE inflammatory reaction continues to be a centre of interest in the medical sciences, largely because of the high incidence of inflammatory diseases throughout the world. One in three people in the West can expect to suffer from some sort of chronic inflammatory disorder during the course of their life. This book, however, is devoted essentially to the acute inflammatory reaction and here Professor Movat has dealt very thoroughly with the subject. He makes no apology for his somewhat idiosyncratic approach, drawing heavily upon his own research findings to illustrate and explain the events of inflammation. This is not to say that the book lacks objectivity. It is an impressive review of current knowledge, with an extensive bibliography that takes up more than a fifth of the text.

The study of inflammation was for many years considered the province of pathologists and has only recently been hijacked by pharmacologists and immunologists. Professor Movat emphasizes the need for integration of the disciplines concerned, but, as a pathologist himself, he is well placed to lead his readers through the historical developments and on to current ideas. Not surprisingly then, he places inflammation in the context of the structure and function of the tissues affected. Beginning with the anatomy of the microcirculation, he goes on to describe the vascular and lymphatic changes occurring as a result of inflammatory injury. The morphological and functional aspects of leukocyte activation are dealt with in a similar way. This approach provides a good basis for the chapters on mediators and mechanisms.

The longest chapter, occupying almost a quarter of the book, tackles the formidable list of mediators that are known to contribute to the vascular phenomena of acute inflammation. Another chapter deals with substances which stimulate leukocyte emigration. The problem with categorizing inflammatory mediators is that they are rarely involved in discrete functions. For example, the prostaglandins, leukotrienes and complement-derived factors contribute to vascular as well as cellular responses in inflammation. Professor Movat has overcome this difficulty by demonstrating the relative importance of individual mediators in processes such as vasodilation, vascular permeability, chemotaxis and degranulation. This has not been achieved without cross-referencing between chapters, which in places is confusing. The picture that emerges, however, is that inflammatory reactions are initiated and amplified by the actions and synergistic interactions of a large number of mediators.

It is a little disappointing that the mechanisms of chronic inflammation are not touched upon until the penultimate chapter of the book. Also, discussions of the treatment of inflammation or the mechanisms of anti-inflammatory drugs have been deliberately avoided. Nevertheless, this is the most comprehensive and up-to-date text available on the subject of acute inflammation, and as such it will prove useful for reference. A large part of the book is based on experimental findings and this aspect should appeal to the post-graduate and post-doctoral workers in inflammation research. Professor Movat should, therefore, succeed in his objective of stimulating the young investigators to whom this monograph is primarily addressed. □

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Engineered viruses out of doors

D.H.L. Bishop

Genetically Altered Viruses and the Environment. Edited by Bernard Fields, Malcolm A. Martin and Daphne Kamely. Cold Spring Harbor Laboratory: 1986. Pp.362. \$63.

THE use of genetically altered viruses in medicine or veterinary practice is, of course, old hat. Since the late eighteenth century and Jenner's pioneering work on smallpox, attenuated genetic variants of disease-causing viruses have been employed to protect man and domestic animals from infection by virulent viruses.

Such vaccine strains of viruses have been obtained by serendipity. What then is the concern about genetically altered viruses in the environment? With modern technologies and precise knowledge of the genetics of viruses (genome sequences and coding strategies), not only can we now achieve the objectives of earlier scientists by direct and specific genetic engineering, but we can go much further in custom-designing viruses for new purposes. It is against this background that the symposium that spawned this book was organized. The issues raised are both broad and far-reaching, and are of importance not only to scientists but also to the public, especially local and national regulatory authorities.

The initial chapters of the book describe the US legislative and regulatory frameworks pertaining to the use of genetically engineered viruses. Corresponding frameworks in other countries are not considered, limiting the interest of this information to the international audience, though one appreciates from these accounts the problems that have arisen in the United States where regulations are being applied to a subject that goes beyond the concepts involved in originally formulating the legislation.

The development and use of genetically engineered viruses in agriculture and forestry will open up new tracts of science, and will doubtless have irreversible environmental consequences. The question of risk assessment is therefore a central one, but there is only restricted discussion of this subject in the book. It was, for example, a major oversight of the organizers that there is a detailed report neither of the use in Australia of the South American myxomatosis virus to control rabbit populations, nor of the domino effects of the introduction of the virus to continental Europe and the United Kingdom. Likewise our extensive knowledge of the use of viral insecticides to control pests of agriculture and forestry is given

short shrift. Chapters are devoted to the subject of virus spread between hosts by vectors, or water or airborne transmission, topics which bear strongly on potential environmental effects. But there is much more to the issues involved than can be covered in each of these short contributions.

In a section on virus tropisms, the genetic basis of the cell-organ selectivities exhibited by certain viruses is discussed succinctly, as is the role of cellular receptors in determining particular virus tropisms and the penetration processes used by viruses to invade cells. Similarly, the genetic elements involved in the control of virus replication and expression of particular viral phenotypes are well reviewed. The complicated matter of virus-host interactions is covered in four chapters dealing with virus persistence and variation, and host responses to virus infection, with particular reference to genetically altered viruses such as the attenuated virus vac-

cines. The final section of the book is devoted to accounts of virus vectors that are of value for the expression of foreign genes (vaccinia, bovine papillomavirus, adenoviruses, *Autographa californica* nuclear polyhedrosis virus). This is a topical subject, in that such vectors are being developed by the biotechnology industry for the production of foreign gene products. Again, however, there is little mention of their use (and impact) in the environment.

All in all, it is evident that the original meeting was a thought-provoking and worthwhile exercise. Unfortunately, the resulting book provides only sketchy outlines of selected areas of the subject matter, and I would question its value to either the specialist or the more general reader. □

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A manifold mind

John C. Marshall

The Citadel of the Senses and Other Essays. By Macdonald Critchley. *Raven*: 1986. Pp.277. \$32.50.

MACDONALD Critchley is one of our most senior and distinguished neurologists, and a man for whom "retirement" means an opportunity to work harder than ever. His current collection of essays, some reprinted, many previously unpublished, bears witness to an undimmed interest in all aspects of neurological disease and an acute awareness of the rich variety of humankind, its triumphs and its foibles. The volume is appropriately prefaced by a novelist's *bon mot*: "Somerset Maugham regarded old age as the time to undertake those tasks shirked in youth because they would have taken too long".

The title essay, "The Citadel of the Senses: The Nose as its Sentinel", restores to olfaction the high ground usually occupied by the visual and auditory systems. Some brief remarks on the comparative anatomy of the nose introduce a vignette on the role of smell in medical diagnosis; but lest such odours seem unduly fetid, we are also treated to extracts from "Vins et Vignes de France", "La Psychologie du Parfumeur", and the adventures of Flush amidst the redolent aromas of spring in Florence. The protean world of touch is equally well represented in a chapter on haptic perception; here Critchley ranges from the skills of the silk mercer, through the neuropsychology of braille reading, to disorders of tactile discrimination and localization consequent upon damage to the peripheral and central nervous system.

Disorders of the visual system likewise provoke a wealth of anecdote and differential diagnosis: in what terms should we describe and explain how some patients with adequate sight nonetheless fail to recognize objects (visual agnosia), or how yet other patients apparently cannot see two objects at the same time (simultanagnosia)? What are we to make of the auras in migraine or the bizarre visual hallucinations that may precede an attack? Was the terrifying abyss that Blaise Pascal purportedly saw plunging to his left a symptom of ophthalmic migraine? (Neurologists are almost as keen as psychoanalysts to diagnose the famous dead.)

Although most of the papers collected here are essays for the left hand, intended to stimulate thought rather than solve problems, one does occasionally long for a more extended analysis. For example: Critchley claims that "it is a vain pursuit to seek to isolate various subtypes of visual agnosia, e.g., for colours or for faces". Yet there are many well-documented case-reports, with extensive testing and more than adequate controls, that do seem to indicate the existence of high-level perceptual disorder in which an impairment of colour or face recognition is disproportionately severe. What *specifically*, one wants to know, does Critchley find inadequate about the investigation and interpretation of these cases? Similarly, in his discussion of claims that there are subtypes of specific developmental dyslexia, Critchley writes: "To medical men like myself such efforts are not impressive and seem premature and speculative". Those of us who have attempted to provide solid, empirical evidence for a typology of dyslexic impairment would welcome criticism more detailed than the blank assertion that "to make a rigid

parcellation within the clinical picture of developmental dyslexia is going too far".

Throughout the volume Critchley wears his prejudices and his enthusiasms on his sleeve. Particularly generous and appealing are his biographical sketches of esteemed neurologists: Hughlings Jackson, Newman Neild and Josef François Babinski. How refreshing it is to discover that the last of these was a person, not merely a reflex. I was especially happy to learn of Babinski's term for "confused, obscure,



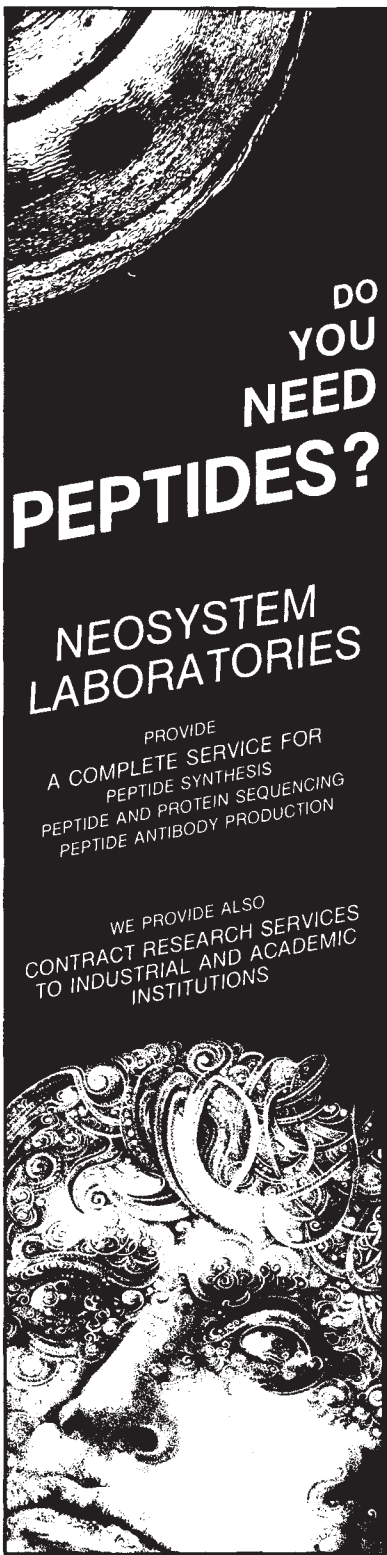
From *The Citadel of the Senses*

Sole stimulation — the Babinski sign, reported in C.r. Séanc. Soc. Biol. in 1896. "Here at last", writes Critchley, "was a clinical shibboleth which would distinguish the organic from the hysteric, the genuine from the counterfeit".

or imprecise" writing — "chieurs d'encre" (ink-shits, as Critchley forthrightly puts it). The enthusiasms that Critchley recommends for study by the young neurologist include the physiology of laughter and smiling, the transcendental states that music can provoke, and the sensory skills of the blind; on occasion an enthusiasm and a prejudice will interact, as when Critchley advocates the study of aphasia in African, Asian and Amerindian languages, but tells the student to take no notice of "contrastive linguistics"!

One final foible: in this, as in most of his collections, Critchley refuses to give references, a habit that is decidedly unhelpful to those whose knowledge and memory cannot match Critchley's own. As it happens, I do have the "original" reference for "Anton's syndrome" (1899), a condition where a cortically-blind patient denies his blindness. But I'd appreciate chapter and verse for the claim that the circumstance "had been described with the utmost lucidity by Seneca centuries ago". □

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Whales: transition in translation

Samuel A. McLeod

General Features of the Paleobiological Evolution of Cetacea. By G.A. Mchedlidze. *Balkema: 1984. Pp. 139 + plates. Dfl. 65, £16.50, \$26.*

FOR the general reader there is, surprisingly, still no overview of cetacean evolution to replace Kellogg's 1928 *History of Whales*. Despite its title, Mchedlidze's book will not suffice as a successor to Kellogg's work. Specialists, on the other hand, will find this to be a very useful volume, though they will also need access to Mchedlidze's 1970 monograph (published in Russian) and his 1977 English-language summary of some of his ideas on cetacean evolution. Mchedlidze's monographs are separate publications, not generally available; this translation provides a much-needed introduction to some of the Soviet fossil cetacean faunas (including important Azerbaidzhan and Georgian fossils discovered over the past two decades), together with several rather idiosyncratic essays on particular morphological transformations.

The descriptions of the fossil cetaceans alone make the book invaluable, though a couple of them are confusing. *Oligodelphis azerbaijanicus* and *Kelloggia barbarus* were both originally described in a 1968 manuscript by Mchedlidze and his erstwhile Russian collaborator, S.M. Aslanova, but by the rules of zoological nomenclature those species names are available only as of 1976. *Kelloggia barbarus* is also said to have "intercostal plates", reminiscent of the now-disproven cetacean "armour" of Küenthal and Lydekker. In 1970 Mchedlidze described a new genus and species, *Mirocetus riabinini*, but in the 1976 work, and this translation of it, the species name appears as *M. riabinini*.

The drawbacks of the book for the unwar reader are several. For example, while Mchedlidze cites most of the pertinent Western palaeontological research, he seems unaware of, or ignores, the general systematic literature. His systematic methodology is therefore seriously out of date. Although he uses individual characters to assign species to higher taxa, most of these characters are primitive and the assignments are based on the vague notion that the characters "most closely resemble" those of a higher taxon. His conclusions frequently are not borne out by the anatomy of the specimens in the photographs. Thus he describes *Ferecetotherium kelloggi* and assigns it to the Aetiocetidae (here placed in the Archaeoceti, although the family is now usually classified in the

Mysticeti), and considers it transitional to mysticetes. But there is no derived character, beside the supposed lack of a mandibular symphysis (not demonstrable from the plate), that this species shares uniquely with any archaeocete or mysticete. It may be a sperm whale. Many of his interpretations about the origin of mysticetes and the evolution of cetacean dentitions rely heavily on the systematic position of this species.

Mchedlidze initially cites the age of all the Russian fossil cetaceans as Upper Oligocene, but notes they are of different evolutionary stages. Subsequently he mentions that most of the specimens were not found *in situ* and that they may actually be of different ages. This problem also affects his systematic conclusions in terms of which taxa might be ancestral to other cetaceans. A different sort of chrono-evolutionary difficulty appears when Mchedlidze uses the old and extreme gradualistic argument that there was not enough time for the evolution of the various cetacean groups from their closest but more primitive reputed relatives. Most particularly, he believes the direct ancestors of cetaceans are "archaic [presumably Late Cretaceous] placental mammals".

Many of Mchedlidze's essays are adaptive scenarios for the origin and evolution of various cetaceans. This is a thought-provoking approach, but it depends directly on the soundness of the systematic conclusions; these, in my opinion, are the book's greatest failing. Several bizarre ideas, some initially espoused by such zoologists as W. Küenthal, R. Lydekker and A.B. Howell, are re-cycled. Noteworthy is Mchedlidze's resurrection of the idea that odontocete skull asymmetry evolved because of increased swimming speed. More astounding is his statement that the dorsal position of the external nares allows cetaceans to remain "out of sight of predators for longer periods of time".

The translator, editor and publisher have done a fine job on the book, correcting some mistakes, adding several text citations not in the original bibliography and, most particularly, reproducing the plates more clearly. The disparity between the number of specimens listed in the legends and those on the plates remains, however, and the genus *Mirocetus* appears in three places instead of the original *Microcetus*, which changes the author's meaning.

This book is indispensable for the specialist. It will also be of interest to Western zoologists more generally, but they should treat its conclusions with great caution. □

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Tip splitting without interfacial tension and dendritic growth patterns arising from molecular anisotropy

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Two growth mechanisms of considerable recent interest are related to a single statistical mechanical model. Tip splitting without interfacial tension occurs when a fluid pushes into another miscible fluid of higher viscosity. Dendritic growth occurs when anisotropic molecules aggregate—a common example is the snowflake. We find that both structures are fractal objects, and can be obtained from a single statistical mechanical model, implying that there is a relation between the underlying physical processes involved.

GROWING structures have fascinated mankind for centuries, and today the field of growth phenomena elicits interest from many disciplines, ranging from medicine and biology to fluid mechanics. Two growth forms that have attracted recent interest are the following:

(1) Dendritic growth¹⁻⁹. No two snowflakes are identical; each is assembled by the random aggregation of water molecules. Yet every child can distinguish a snowflake from other growth forms. The key scientific question is by what mechanism the anisotropy of a water molecule becomes amplified from its weak 'local' effect at the molecular level to its pronounced 'global' effect at the macroscopic level of the snowflake.

(2) Tip splitting¹⁰⁻²⁰. A classic experiment in fluid mechanics concerns the splitting of a low-viscosity body of fluid which results when it is forced under pressure into a high-viscosity fluid. If the two fluids are immiscible, then the interfacial tension between them serves to establish a length scale at which tip splitting occurs. When the two fluids are miscible there is no interfacial tension, yet tip splitting nonetheless occurs. Thus an important question concerns the physical mechanism which determines the point at which the finger splits.

The scientific questions in (1) and (2) have been the object of research for many years, in part because our present state of understanding is so incomplete²¹ that even a little progress would be valuable. The two categories of growth mechanism (1) and (2) have been considered to be quite different, in the sense that the physical basis for one has no relation to that of the other. Here we develop a statistical mechanical model which incorporates both dendritic growth and tip-splitting, thereby relating two disparate fields of enquiry.

Relation between noise and tip splitting

The model is most clearly explained if we begin with the dielectric breakdown model (DBM) of Niemeyer *et al.*²² on, for example, a triangular lattice. We first place a seed particle at the origin of a large circular domain of radius R . If we think of this seed particle as being the source of a fluid of infinitesimally small viscosity, which is being forced under pressure to displace a fluid with much higher viscosity^{23,24}, then the interface must move according to Darcy's law:

$$v_n = -\hat{n} \cdot \nabla P \quad (1)$$

Here v_n is the velocity component normal to the interface, $\hat{n}$ is the normal unit vector and P is the pressure field. P is constant in the less viscous fluid and, because $\nabla \cdot \mathbf{v} = 0$, P satisfies the Laplace equation

$$\nabla^2 P = 0 \quad (2)$$

in the more viscous fluid. Hence the relevant boundary conditions are $P(r, \theta) = 1$ anywhere in the low-viscosity body of fluid, and $P(R, \theta) = 0$ along a circle of radius R .

In a perfect medium with radial symmetry and no pressure fluctuations, the interface will spread out in concentric circles. However, because there is always some noise in the system, a fluid-dynamical instability²⁵ will occur and irregularities in the interface will grow. This noise phenomenon is reproduced in the DBM, which includes fluctuations by means of the following algorithm. First, ∇P is calculated at every perimeter site of the cluster; this is done by solving equation (2) with an overrelaxation technique. At step 1 there is a single seed on a triangular lattice with six perimeter sites. As all sites have equal values of ∇P , the first perimeter site is mapped to the numerical interval $[0, \frac{1}{6}]$ the second to $[\frac{1}{6}, \frac{2}{6}]$, the third to $[\frac{2}{6}, \frac{3}{6}]$ and so forth. Next, a random number generator is used to choose a number in the interval $[0, 1]$. Suppose that this random number is 0.2603238: the second perimeter site is then occupied, and the procedure iterated. For this two-site cluster, ∇P is calculated at the eight perimeter sites, the values are normalized to unity, a new random number is chosen, and one of the eight sites occupied. Such a DBM cluster is characterized by a high degree of noise: as each growth step is determined by only one random number, it is always possible that the random number chosen corresponds to a perimeter site with an extremely small value of ∇P , which by equation (1) should almost never grow. Thus the DBM violates the fundamental Darcy law due to the noise inherent in the algorithm.

We now describe a procedure whereby this noise can be systematically reduced in a controllable fashion. Clearly we need an algorithm such that perimeter sites with extremely small values of ∇P are extremely unlikely to be chosen. This is accomplished by advancing to a new perimeter site only after it has been chosen s times, where s is a parameter which can be tuned. Each perimeter site has a counter which registers how many times that particular site has been chosen. As $s \rightarrow \infty$, the growth of the interface will approach Darcy-law growth, in which any point of the interface grows according to the true local pressure gradient. In the Darcy 'zero-fluctuation' or 'mean-field' limit²⁶ ($s \rightarrow \infty$), the interface would be a perfect circle if there were no underlying lattice.

Figure 1a-c shows the results of calculations for successive values of s . We find that Fig. 1b and c resemble tip splitting as observed in the viscous fingering of both newtonian (refs 19, 27; J. D. Chen, personal communication; R. Lenormand, personal communication) and non-newtonian^{10,12,18} fluids. When $s = 2$ (Fig. 1a), the structure resembles the DBM both qualitatively (although the branches look thicker) and quantitatively

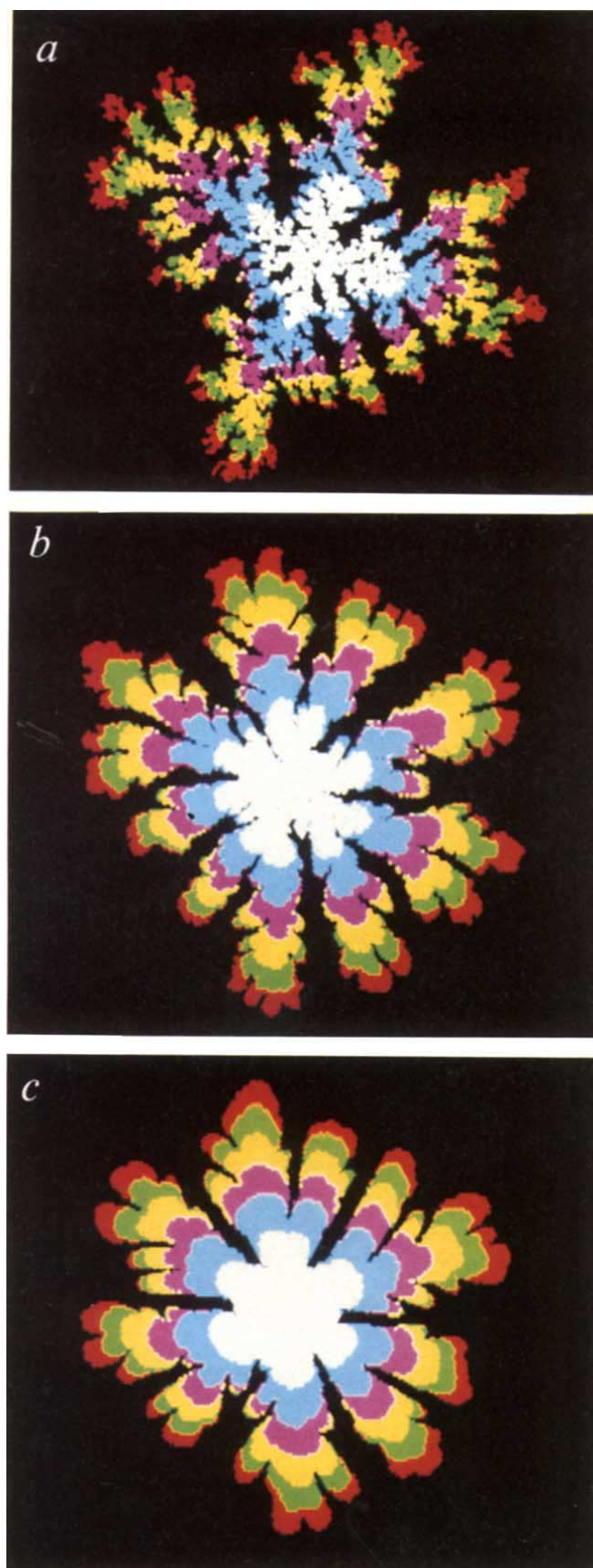


Fig. 1 Examples of fractal structures generated when the anisotropy parameter k is held fixed at unity, but the noise parameter $1/s$ is decreased. In *a*, *b* and *c*, $s=2$, 20 and 200, respectively. For all finite values of s , we find that the fractal dimension is equal to the DBM value, $d_f=1.7$, providing we take care to extrapolate the apparent mass dependence of d_f to its asymptotic limit. The colour coding is as follows: the first one-sixth of the sites are white, the next sixth are blue, followed by magenta, yellow, green and red.

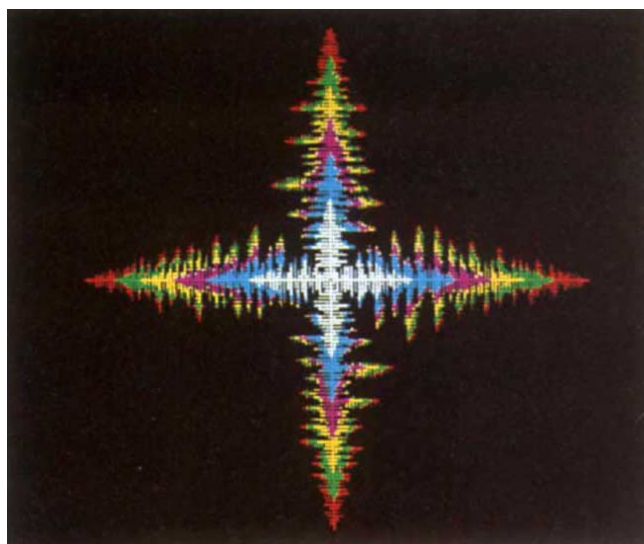


Fig. 2 A typical fractal structure on a square lattice with $s=50$ and a microscopic anisotropy (defined by equations (6), (7)) of $k-1=10$. The colour coding is the same as in Fig. 1.

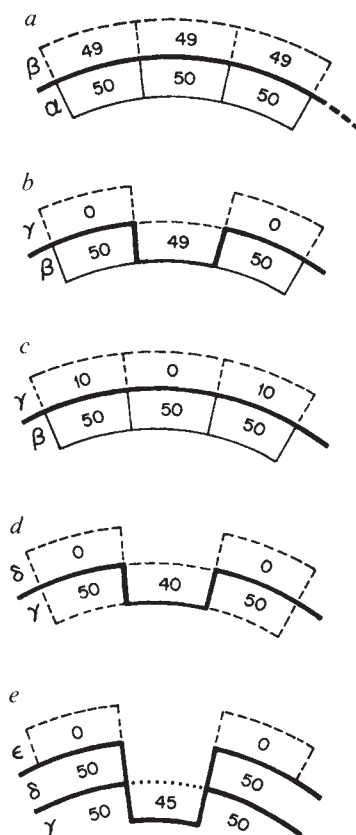
($d_f=1.7$). Here d_f is the fractal dimension obtained, for example, from the slope of a log-log plot of the mass against the caliper diameter. For large s (for example, $s=20$; Fig. 1*b*), the qualitative appearance appears to differ: the system appears at first sight to cross over to a new 'universality class', with a larger value of d_f . However, when we extrapolate the apparent fractal dimension to large cluster sizes we find that $d_f=1.7$ for all values of s ; that is, the growth forms are quantitatively identical, independent of the degree of noise reduction.

Note that tip splitting always occurs by the same mechanism. First a cluster grows 'smoothly', without tip splitting. However, as the radius of curvature increases, the interface becomes 'rough', with both positive (outward) and negative (inward) fluctuations. The positive fluctuations are not significant, as they are soon damped out; however, the negative fluctuations persist (Fig. 3). This is because, for a charged fractal object, the electric field inside a single notch is very small, and the equation relating the electric field to the gradient of the potential is formally identical to the Darcy law relating growth velocity to the gradient of the pressure. Hence, the tiny notch is not likely to be filled in so quickly as one would expect if interfacial tension were present (Fig. 3*d*). The tiny protrusions on both sides of the notch see a much larger field than does the notch, so they attract mass. The tiny notch thus becomes the terminus of a long fjord (Fig. 3*e*). A fjord is almost perfectly screened, and so is almost never filled in. In Fig. 3, $s=50$. If $s>50$ (less noise), then the same tip-splitting mechanism will apply but a negative fluctuation (notch) will decay more efficiently: the system is less susceptible to negative fluctuations and a fjord is formed only when the cluster has reached a larger radius of curvature.

Although the asymptotic fractal dimension d_f is independent of s , the finger thickness W_f clearly increases with s . Moreover, our model explains the existence of a well-defined W_f : the less the noise, the thicker the finger (see Fig. 1). We find the quantitative law:

$$W_f \approx 4.5 \log s + 2 \quad (3)$$

Fig. 3 Schematic illustration of the difference between an outward ('positive') and an inward ('negative') interface fluctuation. A positive fluctuation tends to be damped out rather quickly, as mass quickly attaches to the side of the extra site that is added. On the other hand, a negative fluctuation grows, in the sense that mass accumulates on both sides of the tiny notch. The notch itself has a lower and lower probability of being filled in, as it becomes the end of a longer and longer fjord. This is the underlying mechanism for the tip-splitting phenomenon when no interfacial tension is present. *a* shows the advancing front (row α) of a cluster with $s = 50$. The heavy line separates the cluster sites (all of which were chosen 50 times) from the perimeter sites (all of which have counters registering less than 50). In *a*, no fluctuations in the counters of these three sites have occurred yet, and all three perimeter counters register 49. *b* shows a negative fluctuation, in which the central perimeter site is chosen slightly less frequently than the two on either side; the latter now register 50, and so they become cluster sites in row β . The perimeter site left in the notch between these two new cluster sites grows much less quickly because it is shielded by the two new cluster sites. For the sake of concreteness, let us assume it is chosen 10 times less frequently. Hence by the time the notch site is chosen one more time, the two perimeter sites at the tips have been chosen 10 times (*c*). The interface is once again smooth (row γ), as it was before, except that the counters on the three perimeter sites differ. After 40 new counts per counter, the situation in *d* arises. Now we have a notch whose counter lags behind by 10, instead of by 1 as in *b*. Thus the original fluctuation has been amplified, due to the tremendous shielding of a single notch. Note that no new fluctuations were assumed: the original fluctuation of 1 in the counter number is amplified to 10 solely by electrostatic screening. This amplification of a negative 'notch fluctuation' has the effect that the tiny notch soon becomes the end of a long fjord. To see this, note that *e* shows the same situation after 50 more counts have been added to each of the two tip counters, and hence (by the 10:1 rule) 5 new counts to the notch counter. The tip counters therefore become part of the cluster, but the notch counter has not yet reached 50 and remains a perimeter site. The notch has become an incipient fjord of length 2, and the potential at the end of this fjord is now exceedingly low. Indeed it is quite possible that the counter will never pass from 45 to 50 in the lifetime of the cluster. In our simulations we can see tiny notch fluctuations become the ends of long fjords, and all of the above remarks on the time-dependent dynamics of tip splitting are confirmed quantitatively.



We also find that W_f is independent of the magnitude of the pressure field. To see this, we varied the global pressure gradient by changing the size of our computational grid from 200 to 25 units and found no variation of the finger thickness. This discovery is explained if one considers that the ratio of the local pressure gradient between a site at a finger tip and a site within a fjord does not change if the global pressure gradient is changed: this in turn is a direct consequence of the fact that the pressure field satisfies a Laplace equation.

Previous work on tip-splitting phenomena has focused on explaining the non-zero value of the finger thickness W_f arising from the presence of interfacial tension σ (ref. 28; see also ref. 29). However this explanation cannot be applied to miscible fluids, such as those used in recent experiments^{10,12,18,27,28}, because in this case, by definition, $\sigma = 0$. In our model interfacial tension does not exist (that is, σ acts only on the length scale of a single fluid element or 'pixel'); our observed finger thickness is thus related solely to the concept of noise.

Before proceeding further we note that in the limiting case $s = 1$ the DBM is equivalent to diffusion-limited aggregation³⁰⁻³² (DLA). The diffusion analogue of the DBM for $s > 1$ is a DLA-type model in which growth occurs only after a perimeter site has been hit by s random walkers. If all the counters are reset to zero after each growth step, then we have the Meakin model³³ or the Kertész-Vicsek model⁸, in which growth of the positive fluctuations (the tips) is amplified because a tip of size 1 pixel is more likely to experience the next growth event—so the apparent value of d_f decreases toward unity as the cluster grows. The DLA-type analogue of our DBM-type model in which the counters are not reset to zero after each growth event is the Tang model²⁶, for which it is not the positive fluctuations (the tips) that display amplified growth but the negative fluctuations (the notches). Amplified growth of negative fluctuations is the characteristic feature of DLA, explaining our result that d_f has its DLA value for all finite values of s .

Thus we conclude that noise reduction—arising from sup-

pression of fluctuations—does not change the overall 'universality class', but does introduce a characteristic finger thickness.

Local anisotropy and dendritic growth

Real growth phenomena are never perfectly isotropic. In fact, anisotropy appears to dominate dendritic crystal growth; thus, for example, a snowflake is recognized by its six-fold anisotropy, although the noise is also reflected in the variability from one snowflake to another⁹. No two are alike, although the eye immediately recognizes the pattern of a snowflake.

The problem of understanding the growth of a snowflake has a rich history. A large class of models has focused on introducing anisotropy in a 'global' or macroscopic fashion by introducing angular variables and assuming that the growth depends sensitively upon these variables²⁻⁴. Although the resulting patterns have, by virtue of their rules of construction, the requisite six-fold symmetry, their resemblance to real snowflakes is not striking. Moreover, they lack the random variations that seem to characterize real snowflakes and also fractal objects.

A snowflake grows by successive landings of water molecules, and we have therefore focused our attention on how microscopic irregularities in the landing surface can be translated into the macroscopic structure of the snowflake. To reflect the presence of these microscopic irregularities, we must incorporate into our model the essential fact that the landing sites seen by an incoming molecule are not all equivalent. Hence we replace equation (1) by

$$v_n = -\mathbf{n} \cdot (k \nabla P) \quad (4)$$

where the conservation of mass condition $\nabla \cdot \mathbf{v} = 0$ implies that equation (2) is replaced by

$$\nabla \cdot (k \nabla P) = 0 \quad (5)$$

with the same boundary conditions as for $k = 1$. Here the anisotropy parameter $k = k(x, y)$ would be the permeability in a fluid problem.

Consider a square lattice. One simple choice for $k(x, y)$ is (see Fig. 2)

$$k(x, y) = 1 \quad (6)$$

for x or y even,

$$k(x, y) = k > 1 \quad (7)$$

otherwise equations (6) and (7) express mathematically the fact that the surface affinity for incoming water molecules depends on the spatial coordinate: the incoming particles do not see a perfectly smooth and homogeneous 'landing surface'.

Moreover, our anisotropy is fundamentally different from that considered in, for example, refs 4 and 11. Schematically, in these models the interface is moved according to the rule

$$u = f(\kappa) - f(\theta)u_n \quad (8)$$

where u is the growth velocity, $f(\kappa)$ is an interfacial tension term and u_n is essentially the local pressure (or temperature, or concentration) gradient at the interface. The function $f(\theta)$ indicates the extent to which the growth is enhanced along directions separated by an angle θ . In marked contrast, our model assumes that the anisotropy is present on a molecular level at the interface. We assume that along the interface, the affinity for an incoming water molecule alternates from site to site:

$$u = -f(x, y)u_n \quad (9)$$

We believe that our model is more realistic, as an incoming water molecule in snowflake formation cannot possibly sense the angle $\theta = \arctan(y/x)$, but does see a 'landing surface' whose 'attraction' fluctuates from point to point.

Next we consider the effect of tuning the anisotropy parameter k . Figure 5a-d shows structures grown with a succession of increasing values of k , ranging from 1.1 to 11. We hold s fixed at the value $s = 50$; if s were too small, then noise effects would complicate visualization of the effect of anisotropy. Figure 5 is for a triangular lattice, for which equations (6) and (7) are replaced by a different rule: we set $k > 1$ for every fifth row of the three principal directions of the lattice (E-W, NE-SW, NW-SE). We see from Fig. 5 that as k increases there is a pronounced change from the isotropic case $k = 1$, and the resulting growth (see, for example, Fig. 5d) resembles a 'snowflake' for reasons more subtle than merely the characteristic 6-fold axis of rotation³⁴. Using standard methods (for example, all three methods of ref. 18), we measured d_f for this 'snowflake' and found values that decrease with the number of particles used in the calculation. Extrapolating to infinite size, we find^{35,36} $d_f = 1.5 \pm 0.1$.

Although the structure at first sight appears to be somewhat ordered, we realize that this is a trick played by the 6-fold axis. In fact, an individual branch is quite disordered, with side branches of all sizes extending from it. The reason $d_f > 1$ is that the side branches occur with many different length scales. This is especially apparent from Fig. 5d, where we see from the colour coding that the latest particle to arrive can attach to the side branches as well as to the tip. Figure 5e shows real snowflakes with side branches, which show a striking resemblance to the anisotropic simulations of Fig. 5d. The differences between Fig. 5d and e are the subject of current investigation.

We now address the actual structure of the fractal objects in the presence of anisotropy. It is important to note that there are distinct effects that cooperate to generate the final structure obtained. The first effect is the fine structure of the side branches (see Fig. 2), consisting of a set of 'trees' of varying height, as shown schematically in Fig. 4a. The trees are mainly without branches, as the anisotropy favours growth only in even-numbered rows or columns of the lattice. However the height of a tree varies widely from one tree to the next, due to the tendency of tall trees to screen shorter trees. An analogous variation in the height of trees has been found by Meakin³⁷ in his classic studies of DLA on a planar substrate: he found that

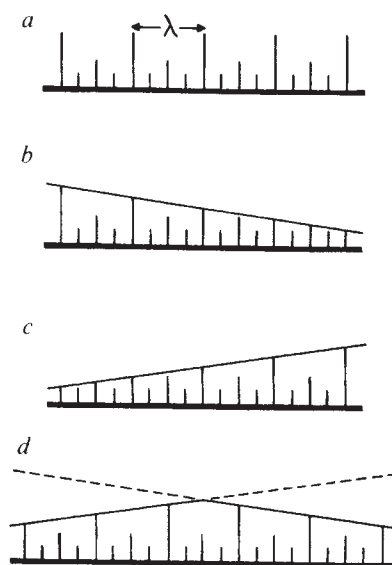


Fig. 4 Schematic illustration to explain the characteristic shape of the four arms of the 'snowflake' cluster in Fig. 2; a recalls the fundamental structure of aggregation onto an equipotential surface, first studied by Meakin³⁷. For simplicity, the 'trees' are drawn as straight line segments, and the hierarchical or fractal distribution of tree height is indicated by a difference of a factor of 2 between successive sizes, together with a spacing, $\lambda(M)$, which increases as M^{1/d_f} , where M is the total cluster mass. b shows the modification expected from the fact that the regions of an arm near the centre have more time to accumulate mass than the regions near the tip. c shows the effect of the fact that ∇P is much larger near the tip; d shows the result of combining a-c, and resembles the overall shape observed in Figs 2 and 5d.

the resulting fractal structure is a 'forest' of trees, with fewer but taller trees surviving at large times due to their tendency to shield the shorter trees.

The main difference between our work and the Meakin (planar substrate) DLA simulations is our lattice anisotropy (parameterized by $k - 1$) and our noise reduction (parameterized by $1/s$), which have the effect of making the trees tall and straight instead of ramified. Consider now the overall profile for the height of the trees in the side branches. This profile can be understood mathematically as arising from the product of two functions. The first, a decreasing function from origin to tip, is related to the fact that the regions of the branches that were formed at early times tend to be larger than the regions of the branches that were formed at late times (Fig. 4b). The second, an increasing function, is related to 'screening'; that is, to ∇P , which is larger near the tips and smaller near the origin (Fig. 4c). As $v \propto \nabla P$, the growth rate is larger near the tips. The product of the increasing and decreasing functions gives the characteristic profile for the height of the trees in the side branches (Fig. 4d).

We also measured as a function of cluster mass: (1) the caliper width of the side branches of Fig. 2, and (2) the caliper diameter of the entire cluster. Both log-log plots are parallel, with slope $1/d_f$.

Discussion

We have shown that two fundamental physical phenomena that are not yet understood, dendritic growth and tip splitting in the absence of interfacial tension, can be related in that both arise from the same statistical mechanical model—a generalization of the DBM²². This means that there are physical features common to both phenomena: they differ only in parameter values. In our model we can incorporate in a direct and systematic fashion the crucial role played by fluctuation phenomena

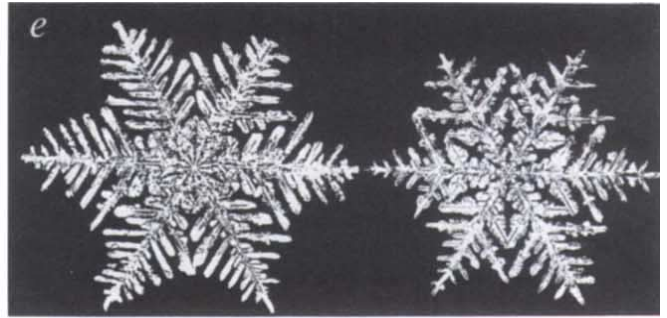
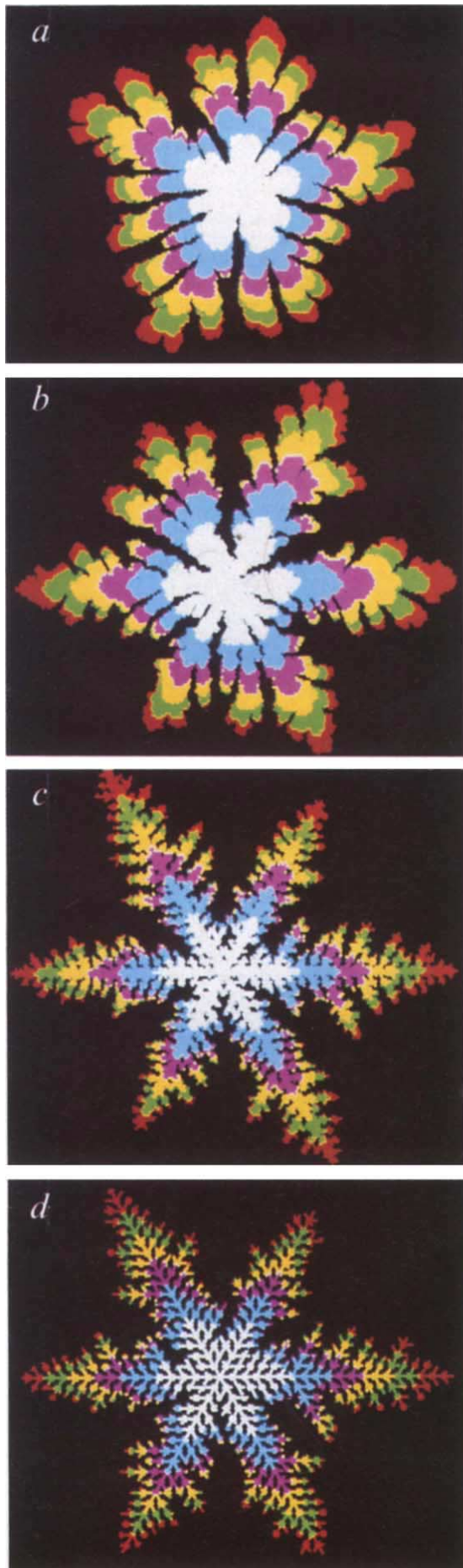


Fig. 5 *a-d*, Examples of fractal structures formed when the noise parameter s is held constant at $s = 50$, but the anisotropy parameter k is chosen to be, respectively, $k-1 = 0.1, 0.31622, 1.0$ and 10.0 (0.31622 interpolates logarithmically between 0.1 and 1.0). The limiting fractal dimension (as $\text{mass} \rightarrow \infty$) is $d_f \approx 1.5$, independent of k , for all $k > 1$. The colour coding is the same as in Fig. 1. *e*, Examples of real snowflakes (reproduced with permission from ref. 9) which show a striking resemblance to *d*.

and anisotropy. The physical picture that we have proposed is embodied in two fundamental equations, (4) and (5) (or (1) and (2) for $k=1$). The second equation describes the spatial change of the pressure field which drives the instability; the first represents the 'growth law', which relates the growth rate of the interface to the pressure field. We have used a generalized Darcy-type law, which enables us to selectively tune both noise and anisotropy.

The overall physical picture that emerges is as follows: Tip-splitting phenomena in the absence of interfacial tension are triggered by microscopic fluctuations (that is, noise). Although positive and negative fluctuations of the interface occur symmetrically, the stability (and hence the subsequent growth) of positive and negative fluctuations are totally different: tip splitting is the direct consequence of this asymmetry in the stability of positive and negative fluctuations. A small protrusion of size 1 pixel is much less long-lived than a small notch of the same size; in fact, it is remarkably difficult to fill even the shallowest notch. Zero noise ($s=\infty$) results in a compact (non-fractal) circular object. A very low noise level (large s) has little effect when a cluster is small, but its effect becomes much more pronounced as the cluster grows larger. In the limit of infinite cluster size, an arbitrarily small but non-zero amount of noise is sufficient to make the cluster fractal. The measured fractal dimension is identical to that of DBM and DLA, two models designed to describe phenomena in the limit in which there is a very high noise level.

The tip-splitting phenomena that occur in the case of zero anisotropy are generalized into a fractal hierarchy of side branches in the presence of anisotropy. In the limit of infinite cluster size even a tiny degree of anisotropy changes the fractal dimension from the DLA value of 1.7 to the value 1.5.

Thus, the complete phase diagram has $1/s$ on the abscissa and $(k-1)$ on the ordinate. Asymptotically we find that d_f is constant, at the DBM value of ~ 1.7 , everywhere on the x -axis, and d_f is also constant, at the value 1.5, everywhere else in the phase diagram except on the y -axis (zero noise), where $d_f = 1$. Thus noise reduction is not a sufficient perturbation to change d_f from its DBM value, because the negative fluctuations persist for all values of s , and these negative fluctuations control the value of d_f . On the other hand, anisotropy at the microscopic level does change d_f . Further details of this phase diagram suggest an intriguing analogy to critical point phenomena, and this will be the subject of future investigation.

Finally, we return to the question posed in the introduction, of how a tiny anisotropy can become 'amplified' from its local effect at the molecular level to a global effect at the macroscopic

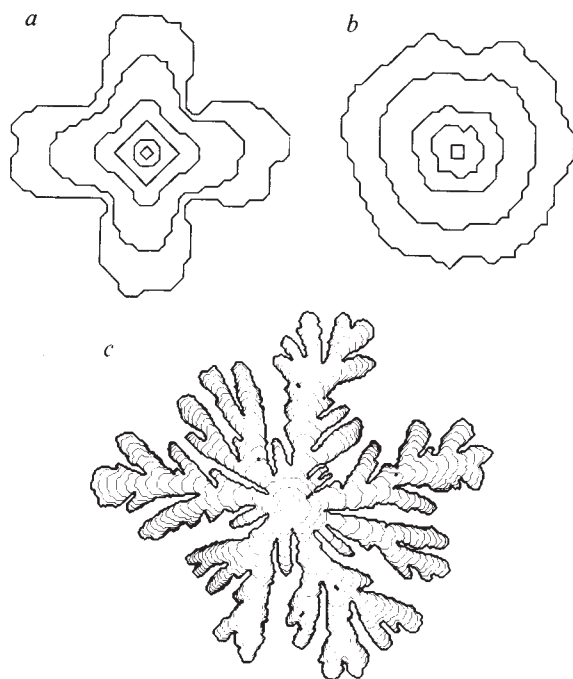


Fig. 6 *a*, Initial growth of an interface on a square lattice for the case $s = 500$. Growth can occur in any of the four space directions (four nearest neighbours). The interface is shown after 5, 21, 85, 200, 500 and 1,000 growth steps. *b*, As in *a*, except that here growth can occur into eight directions (four nearest neighbours and four next-nearest neighbours). The interface is shown after 9, 21, 85, 200, 500 and 1,000 steps. *c*, Viscous fingering structure for $s = 50$, after 15,000 growth steps for eight-fold coordination on a square lattice. The first four contour lines are drawn after 100, 300, 650 and 1,000 steps; subsequent lines are drawn at intervals of 1,000 steps.

level. Our model directly demonstrates this fact: we have shown that in the presence of anisotropy, the resulting fractal dimension is not the DBM value of 1.7, but rather tends asymptotically toward 1.5, a new 'universality class'. Our result is supported by Meakin's very recent calculations for DLA³⁸ without any local anisotropy, except for that arising from the square-lattice substrate. For this classic and well-studied system, 'pure DLA',

Meakin finds the same behaviour that we find in the presence of anisotropy, but only after the cluster size reaches 5 million sites—almost three orders of magnitude larger than the clusters we study! Thus Meakin's local anisotropy, arising solely from the effect of the square lattice itself on the trajectories of random walkers, can lead to a pronounced global effect, altering not only the overall appearance of the cluster but also the fractal dimension itself. As pure DLA is the limit of maximum noise ($s = 1$), we have to wait for an extremely large cluster to see its effect. To support this idea, we systematically reduced s from 50 to 1 while keeping the local anisotropy fixed at the value $k = 2$. When $s = 50$ it is easy to see the snowflake anisotropy pattern, but as s decreases the snowflake vanishes.

To understand better the subtle role played by the anisotropy of the square lattice, we show in Fig. 6*a* the initial growth events for the case $s = 500$, $k = 1$. The early stage of growth is characterized by the competition of 'lattice anisotropy', which attempts to pull the interface into the four principal directions of the plane, and 'interface smoothing' (due to the decay of positive and negative fluctuations), which initially prevents splitting. The competition between these two contradicting tendencies leads to an oscillation of the interface: the structure of the interface alternates between a circle and a diamond-shaped cusp until, eventually, the weak anisotropy of the lattice dominates. As in real systems, no cusp singularities³⁹ occur: the noise in our systems smooths the sharp corners of the initial cusp as interfacial tension would do. A well-defined finger thickness has developed. The larger the value of s , the smaller is the noise and the larger is W_f . To weaken the lattice anisotropy, which results from the rule that growth is possible only in one of the four space directions (nearest neighbours), we can also allow growth into the four diagonal directions (next-nearest neighbours). Figure 6*b,c* shows that such growth is initially almost circular until it reaches a critical radius, after which negative fluctuations are no longer filled in. This gives rise to the characteristic fingering structure shown in Fig. 6*c*. Thus this model seems to represent both qualitatively and quantitatively the viscous fingering phenomenon for the case of miscible fluids (zero interfacial tension).

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Nodule-specific expression of a chimaeric soybean leghaemoglobin gene in transgenic *Lotus corniculatus*

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When a chimaeric soybean leghaemoglobin gene was introduced into the genome of another legume species, Lotus corniculatus, nodule-specific expression of the chimaeric gene was found in root nodules formed on fully regenerated plants inoculated with the Lotus microsymbiont, Rhizobium loti. Expression under control of the 5' upstream region of the soybean gene was regulated at the level of RNA and followed the correct developmental timing. This indicates a conserved induction mechanism for leghaemoglobin genes in legumes.

THE symbiotic association between soil bacteria of the genus *Rhizobium* and plants of the family Leguminosae results in the formation of a unique specialized plant organ—the root nodule. Fixation of atmospheric dinitrogen by the bacterial nitrogenase enzyme within this organ enables the plant to grow without external supply of reduced nitrogen. The interaction between the symbionts is generally very specific and has been used to subdivide the bacterial genus into species and the legumes into cross-inoculation groups¹. *Bradyrhizobium japonicum* will thus infect soybean while *Rhizobium loti* infects *Lotus corniculatus*. Normal root nodule formation occurs only after invasion of the plant root by the compatible bacteria. Successful invasion initiates a series of developmental events in the plant. Root cortex cells re-differentiate into meristematic cells that proliferate and differentiate to form the organized tissues². Around 30 different plant encoded-polypeptides ('nodulins') are specifically synthesized or present in higher concentration during this development^{3,4}. Biochemical function has been assigned to genes encoding glutamine synthetase⁵, uricase^{6,7} and leghaemoglobin polypeptides⁸.

Four leghaemoglobin proteins, Lbc₃, Lbc₂, Lbc₁ and Lba, are synthesized in soybean nodules⁸. The corresponding genes together with a pseudogene and a truncated gene are organized in two clusters with intergenic regions of some 2 kilobases (kb) separating the individual genes^{9,10}. Induction of the four active *lb* genes occurs in a sequential manner during nodule development. Transcription of the *lbc₃*, *lbc₂* and *lbc₁* genes starts slightly before the appearance of visible nodules while transcription of the *lba* gene is delayed for another day or two¹¹. Later in development, transcription of the *lba*, *lbc₃*, *lbc₁* genes is drastically increased at the same time as several other nodulin genes are activated, indicating common features in the regulation of nodule-specific genes¹¹. Typical eukaryotic promoter sequences homologous to sequences required for optimal transcription of a mammalian β -globin gene are located 5' to each of the coding sequences of the four active genes^{12,13}, but β -globin gene expression is also dependent on downstream sequences¹⁴. In order to analyse the role of 5' upstream and 3' downstream sequences in the specific regulation of leghaemoglobin genes, we have transferred a chimaeric gene (*lbc₃ 5'3'-cat*) with the composition 5' *lbc₃*-chloramphenicol acetyltransferase (CAT) coding sequence-3' *lbc₃* to a heterologous legume species, *Lotus corniculatus* (bird's-foot trefoil), using the Ri plasmid of *Agrobacterium rhizogenes* as gene vector.

Here we describe the nodule-specific expression of the chimaeric soybean *lbc₃ 5'3'-cat* gene in root nodules formed on fully regenerated plants of a member of the legume family. We further show that the expression is regulated at the level of RNA and that the correct transcription initiation signals are used. Finally it is shown that the chimaeric gene is activated at the correct stage of nodule development.

Transformation system

The minimal requirement for the study of the expression of transferred genes in root nodules is a legume transformation and regeneration system that allows transformed cells to differentiate into roots. Such a system was developed using the capacity of *Agrobacterium rhizogenes* to induce root formation in transformed plant cells. For this particular study an approach leading to the integration of a complete 'intermediate cloning vector'¹⁵ carrying the chimaeric gene in the T-DNA region of the Ri plasmid was chosen. Figure 1a shows the two intermediate vectors, pAR29 and pAR30, used to integrate the chimaeric *lbc₃ 5'3'-cat* gene into *A. rhizogenes* T-DNA. Homologous recombination through the pRi *EcoRI* fragments 36 and 40 was obtained after conjugation¹⁶ of the two 'intermediate vectors' from *Escherichia coli* to the *A. rhizogenes* 15834 strain¹⁷. Transconjugants underwent Southern blot analysis to demonstrate the presence of the chimaeric gene and of co-integrate formation through the homologous T-DNA sequences (data not shown). Roots formed after wound site infection of *L. corniculatus* seedlings were cultured *in vitro* and freed from the infecting bacteria by treatment with antibiotics (500 μ g ml⁻¹ Claforan). Whole plants were regenerated from root cultures through somatic embryogenesis or organogenesis¹⁸. The structure of the co-integrate T-DNA region transferred to *L. corniculatus* lines L6-23 and L5-9, as deduced from recombinant constructions and confirmed by Southern blot analysis, is given in Fig. 1b. Figure 2 demonstrates the presence of the *lbc₃ 5'3'-cat* gene in the L6-23 line.

The number of T-DNA inserts in the two plant lines were estimated from the number of *EcoRI* or *HindIII* restriction fragments hybridizing to the left T_L-DNA border in genomic DNA Southern blot analysis. An *A. rhizogenes* T_L-DNA-derived probe shown below Fig. 2 was used to recognize the various restriction fragments generated after insertion of the T_L-DNA into the plant genome. Two T-DNA inserts were found in L6-23 while only one insert was present in the L5-9 line. These values reflect the minimum copy numbers for the *lbc₃ 5'3'-cat* gene. Regenerated plants were transferred to growth cabinets and

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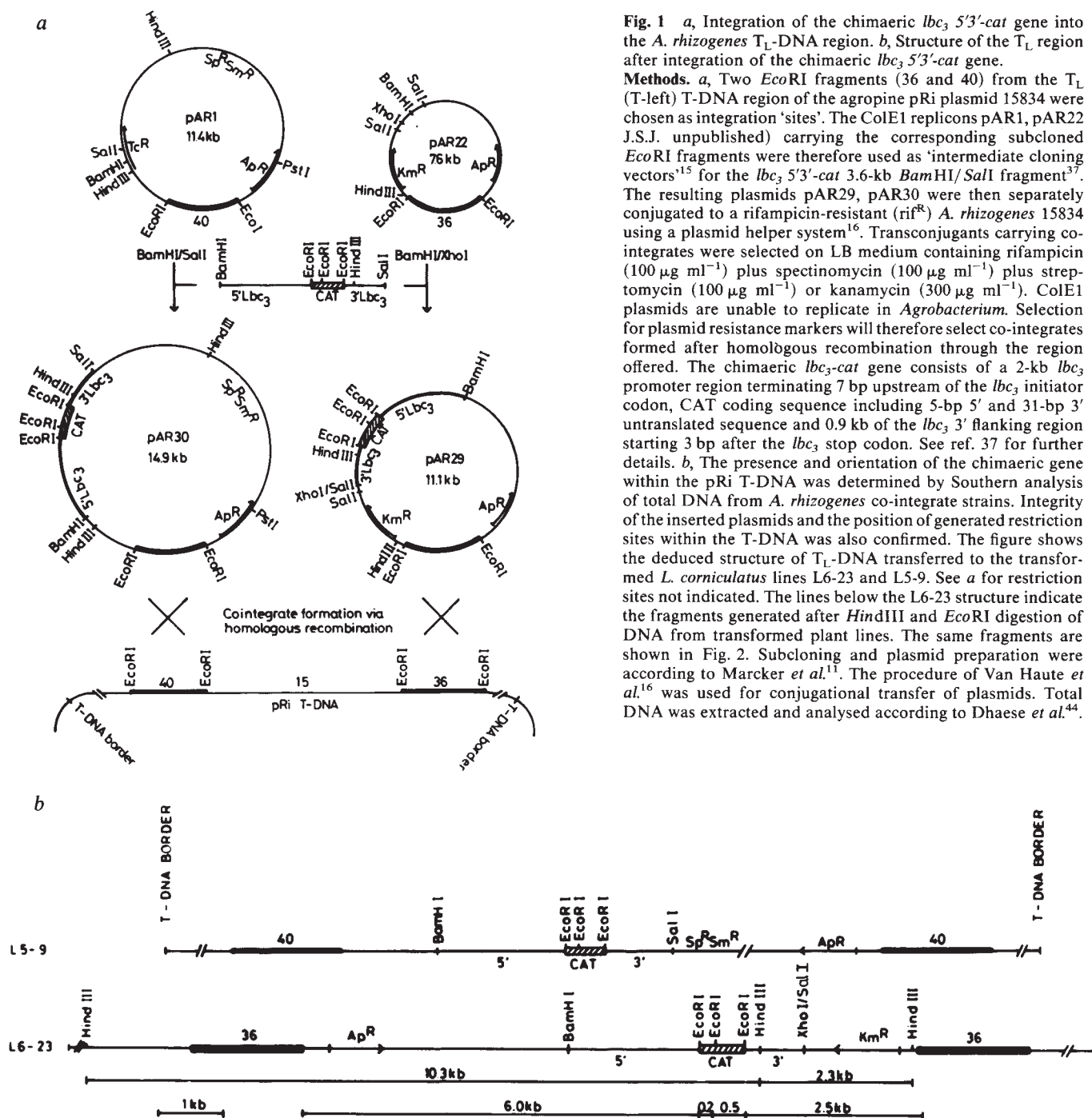


Fig. 1 *a*, Integration of the chimaeric *lbc3* 5'3'-*cat* gene into the *A. rhizogenes* T_L-DNA region. *b*, Structure of the T_L region after integration of the chimaeric *lbc3* 5'3'-*cat* gene.

Methods. *a*, Two *EcoRI* fragments (36 and 40) from the T_L (T-left) T-DNA region of the agropine pRi plasmid 15834 were chosen as integration 'sites'. The ColE1 replicons pAR1, pAR22 J.S.J. unpublished) carrying the corresponding subcloned *EcoRI* fragments were therefore used as 'intermediate cloning vectors'¹⁵ for the *lbc3* 5'3'-*cat* 3.6-kb *BamHI*/*SalI* fragment³⁷. The resulting plasmids pAR29, pAR30 were then separately conjugated to a rifampicin-resistant (*rif^R*) *A. rhizogenes* 15834 using a plasmid helper system¹⁶. Transconjugants carrying co-integrates were selected on LB medium containing rifampicin (100 µg ml⁻¹) plus spectinomycin (100 µg ml⁻¹) or streptomycin (100 µg ml⁻¹) or kanamycin (300 µg ml⁻¹). ColE1 plasmids are unable to replicate in *Agrobacterium*. Selection for plasmid resistance markers will therefore select co-integrates formed after homologous recombination through the region offered. The chimaeric *lbc3*-*cat* gene consists of a 2-kb *lbc3* promoter region terminating 7 bp upstream of the *lbc3* initiator codon, *CAT* coding sequence including 5-bp 5' and 31-bp 3' untranslated sequence and 0.9 kb of the *lbc3* 3' flanking region starting 3 bp after the *lbc3* stop codon. See ref. 37 for further details. *b*, The presence and orientation of the chimaeric gene within the pRi T-DNA was determined by Southern analysis of total DNA from *A. rhizogenes* co-integrate strains. Integrity of the inserted plasmids and the position of generated restriction sites within the T-DNA was also confirmed. The figure shows the deduced structure of T_L-DNA transferred to the transformed *L. corniculatus* lines L6-23 and L5-9. See *a* for restriction sites not indicated. The lines below the L6-23 structure indicate the fragments generated after *HindIII* and *EcoRI* digestion of DNA from transformed plant lines. The same fragments are shown in Fig. 2. Subcloning and plasmid preparation were according to Marcker *et al.*¹¹. The procedure of Van Haute *et al.*¹⁶ was used for conjugational transfer of plasmids. Total DNA was extracted and analysed according to Dhaese *et al.*⁴⁴.

inoculated with the *Rhizobium loti* strain NZP2037¹⁹. Nodules for analysis were harvested 3–6 weeks later.

Nodule-specific expression

Chloramphenicol acetyltransferase activity was measured in 3–6-week-old nodules excised from the roots of L6-23 and L5-9 transformed *Lotus* lines. Root tissue as well as leaf plus stem fragments derived from the same transformed plant were also extracted and *CAT* activity measured. Conversion of ¹⁴C-chloramphenicol to the acetylated forms followed by thin layer chromatographic separation was used to assay *CAT* activity. Data from *Lotus* lines L6-23 and L5-9 are presented in Fig. 3, together with data from untransformed *Lotus* plants and from a line transformed with the complete *lbc3* gene. No *CAT* activity could be detected in any tissue from untransformed or *lbc3*-transformed plants. High enzymatic activity was, however,

expressed from the chimaeric *lbc3* 5'3'-*cat* gene in root nodules of the L6-23 line. Neither root nor leaf plus stem tissue from this same line contained any *CAT* activity. The *lbc3* 5'3'-*cat* gene is therefore expressed in an organ-specific manner. Similar results were obtained with the L5-9-transformed line. A remarkable result is the lack of expression in any of the other major tissues tested. Even prolonged exposure of the autoradiogram did not reveal any *CAT* activity in transformed leaf stem or root tissues. The *CAT* assay conditions used would have detected an activity 5,000 times lower than the activity observed in root nodules.

Expression regulated at RNA level

The nodule-specific expression of *CAT* from the soybean *lbc3* 5'3'-*cat* gene may be the result of transcriptional, translational or post-translational control. In order to distinguish among these

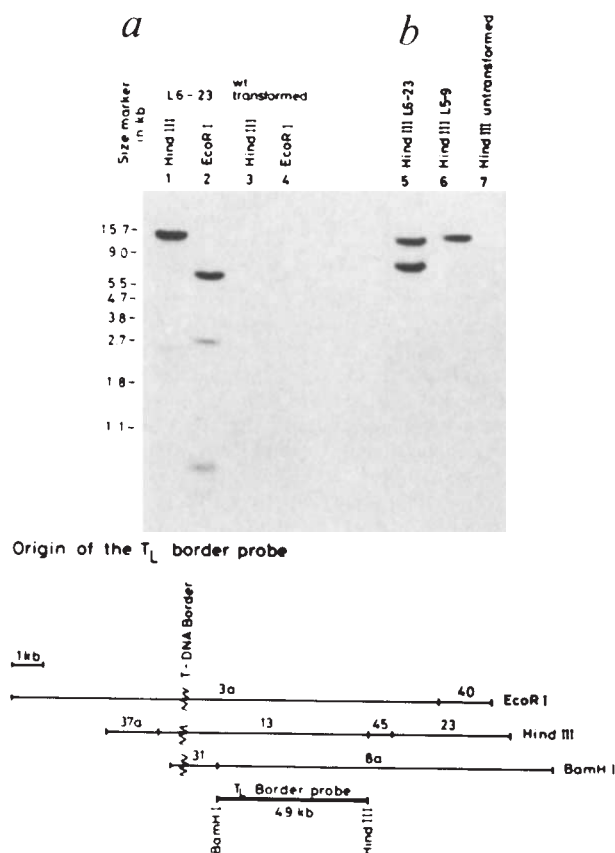


Fig. 2 *a*, Southern hybridization analysis of the transformed *L. corniculatus* line L6-23. Lanes 1 and 2 contain $\sim 10 \mu\text{g}$ HindIII or EcoRI-digested DNA from leaves of L6-23 plants. Lanes 3 and 4 contain corresponding digests of DNA from *L. corniculatus* plants transformed with the *A. rhizogenes* wild-type strain. The size of the fragments hybridizing to a *lbc*₃ 5'3'-cat probe are indicated below the diagram of the co-integrate structure in Fig. 1*b*. *b*, Estimation of copy number of the *lbc*₃ 5'3'-cat gene in L6-23 and L5-9 *Lotus* lines. A BamHI/HindIII fragment adjacent to the left T_L border was used in Southern hybridization to visualize the EcoRI and HindIII fragments generated after insertion of the T_L-DNA into the plant genome. The restriction map⁴⁵ below shows the exact position of the BamHI/HindIII restriction fragment used as probe and give the size of EcoRI, HindIII restriction fragments hybridizing to this probe within the pRi plasmid itself. Lanes 5–7 contain HindIII digests of L6-23, L5-9, and untransformed *Lotus* plants, respectively. Copy numbers were estimated by counting the hybridizing bands. Total plant DNA was extracted and digested according to Dellaporta *et al.*⁴⁶.

possibilities total RNA was extracted from nodule, root and leaf plus stem tissues from L6-23, L5-9 and *lbc*₃ transformed control plants. The RNA was separated on agarose gels, transferred to nitrocellulose and hybridized with a probe from the CAT coding sequence. A strong hybridization signal was detected with L6-23 and L5-9 nodule RNA while none of the RNA preparations from any other tissue tested showed hybridization (Fig. 4*a*). Expression of the *lbc*₃ 5'3'-cat gene is therefore clearly controlled at the level of RNA. The gene for ubiquitin, a histone-associated protein, is constitutively expressed in a range of eukaryotes including plants^{20,21}. Hybridization of a heterologous ubiquitin cDNA to the various RNA preparations was therefore used as an internal standard. Figure 4*b* shows the constitutive expression of endogenous *Lotus* ubiquitin genes in the tissues tested.

Lotus corniculatus plants contain endogenous leghaemoglobin genes, and cross-hybridization with soybean leghaemoglobin

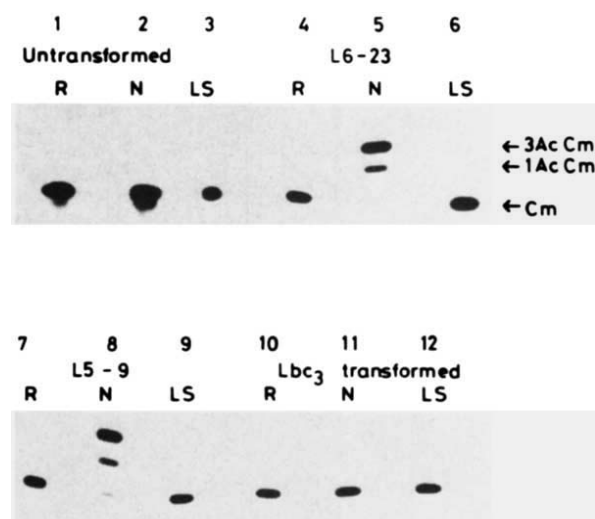


Fig. 3 Expression of the chimaeric *lbc*₃ 5'3'-cat gene in various tissues from transformed and untransformed *Lotus* plants. Equal amounts of tissues were homogenized in a mortar and CAT activity measured by conversion of ¹⁴C-chloramphenicol (Cm) to acetylated (Ac Cm) derivatives. Lanes 1–3 demonstrate the lack of CAT activity in root (R), nodule (N), and leaf+stem (LS) tissue of untransformed plants. Lanes 4–6 and 7–9 show the CAT activity in corresponding tissues of the L6-23, L5-9 *lbc*₃ 5'3'-cat transformed lines. Conversion of chloramphenicol in lanes 5 and 8 demonstrate the organ-specific expression of the chimaeric gene in root nodules. Lanes 10–12 show the assays of *lbc*₃ transformed control tissues. Reconstruction experiments showed that CAT enzyme from root nodule extracts are stable when mixed with root and leaf plus stem extracts (data not shown).

Methods. Tissue was homogenized with mortar and pestle in 50 mM Tris pH 7.5 1 mM EDTA, 400 mM NaCl, 2 mM phenylmethylsulphonyl fluoride buffer (1:1 w/v) at 0 °C. The crude extract (50 μl) was diluted 10-fold into reaction mixture (450 μl) containing 200 mM Tris pH 7.5, 0.5 mM acetyl coenzyme A and 0.5 μCi ¹⁴C-chloramphenicol and incubated for 1 h at 37 °C. Samples were extracted with 5 ml ethyl acetate and analysed by thin layer chromatography using chloroform/methanol (95:5). The chromatograms were autoradiographed and CAT activities quantitated by counting corresponding regions by liquid scintillation.

Standard assays contained 100 μg protein.

sequences was detected in Southern blot DNA hybridizations (data not shown). A hybridization signal was also detected in Northern blots. Figure 4*c* shows the signal obtained with a full-length cDNA probe for the soybean *lba* gene. The steady-state level of transcripts from the chimaeric *lbc*₃ 5'3'-cat gene is more than 200-fold higher than the lowest level detectable with the Northern hybridization technique used here.

The size of the CAT hybridizing transcript is approximately 900 nucleotides, which corresponds well with the 860 nucleotides between the *lbc*₃ cap site and the *lbc*₃ polyadenylation site on the chimaeric gene. The endogenous soybean *lbc*₃ has a cap site 51 nucleotides upstream of the ATG codon²² (position +1). To test whether the heterologous host *L. corniculatus* recognizes and faithfully utilizes the 5' soybean *lbc*₃ promoter, the transcription initiation point of the CAT transcript from L6-23 nodules was determined. Total nodule RNA was subjected to oligo(dT) chromatography and subsequently used in a primer extension assay. An oligonucleotide primer (5'CAACGGTGGTA-TATCCAGTG3') complementary to nucleotides 15–34 in the CAT coding sequence was used. Figure 5 shows the resulting 85, 86, 87, 88 and 90 nucleotide long cDNA species generated. These sizes correspond well with the distance between the cap site (+1) and the 5' end (+83) of the primer. The cDNA species of 85, 87 and 88 nucleotides correspond exactly to endogenous

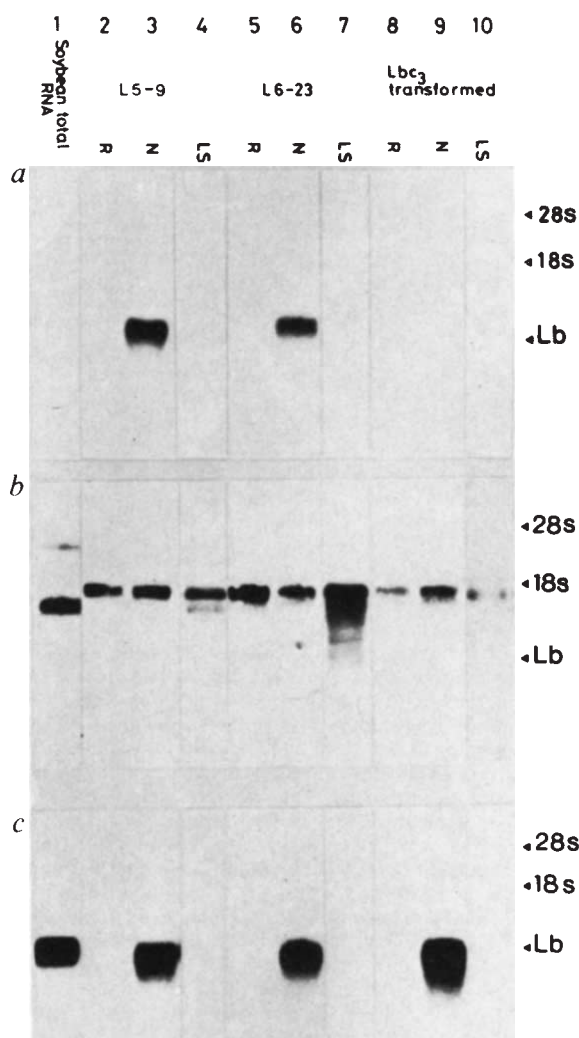


Fig. 4 Northern analysis of transcripts from tissues of *lbc3* 5'3'-cat transformed and *lbc3* transformed *Lotus* lines. Five μ g of total RNA extracted from root (R), nodule (N) or leaf+stem (LS) tissue was separated in formaldehyde-containing agarose gels and blotted onto nitrocellulose (Gene Screen). Lane 1 contains total RNA from 20-day-old soybean nodules. Lanes 2-4 total RNA from roots, nodules or leaf+stem of the *lbc3* 5'3'-cat-transformed L5-9 line. Lanes 5-7 contain total RNA from root nodules, roots or leaf+stem of the L6-23 line while lanes 8, 9 and 10 contain RNA from an *lbc3* transformed line. The complete CAT coding sequence was used as a probe in *a* and demonstrates the presence of the *lbc3* 5'3'-cat transcript in L5-9 and L6-23 nodules. In *b* the transcript for the constitutive ubiquitin genes is visualized using a cDNA probe for the human ubiquitin gene²⁰. Nodule-specific expression of the endogenous *Lotus lb* genes is shown in *c*. A cDNA probe for the *lba* gene was used in this hybridization. Total RNA was extracted and analysed according to Marcker *et al.*¹¹ except for leaf+stem RNA which was extracted in 100 mM Tris pH 8, 50 mM EDTA, 500 mM NaCl, 5% β -mercaptoethanol, 1.4% sarkosyl at 65 °C for 15 min.

soybean *lbc3* cap sites determined by S₁ mapping at lower exonuclease concentrations²².

Developmental regulation

Vertebrate β -globin genes are regulated by a gene-switching mechanism that ensures transcription of only one gene in embryonic, fetal and adult tissue²³. The four active leghaemoglobin genes (*lba*, *lbc3*, *lbc2*, *lbc1*) are also induced at different stages of nodule formation, but all four genes remain active throughout the lifetime of the nodule. The *lbc3*, *lbc2* and *lbc1* are expressed at a low level immediately before the nodule turns pink. At this developmental stage there is a strong and abrupt increase in transcription of the *lbc3*, *lbc1* and *lba* genes. A number of 'nodulin' genes are induced at this stage while the bacterial nitrogenase genes are induced even later¹¹. CAT activity from the chimaeric *lbc3* 5'3'-cat gene in L6-23 was therefore followed throughout nodule development. Figure 6 shows the induction kinetics of the chimaeric gene relative to nodule development and nitrogenase activity. The lack of CAT activity in small white nodules (panel 2 in Fig. 6), the low level of activity in white nodules (panel 3) and the strong increase in activity in pink nodules with high nitrogenase activity follow the kinetics of the native *lbc3* gene. Correct developmental regulation of the *lbc3* promoter is thus maintained in the *L. corniculatus* transformants.

Discussion

Agrobacterium Ti plasmids have been used as gene vectors for plant transformation by a number of investigators. Protoplasts²⁴, leaf disks²⁵, callus²⁶ and wounded plants²⁷ have been infected and transformed tissue was recovered by screening or selection²⁸. The transferred genes are functionally expressed and expression in complete regenerated plants has so far been achieved in tobacco²⁹⁻³⁴, petunia^{31,34-36} and tomato plants²⁵. Chimaeric genes consisting of the T-DNA-derived nopaline synthase promoter and coding sequences specifying resistance to chloramphenicol, kanamycin and methotrexate respectively were initially used for transfer and expression in model studies^{29,32}. More recently, chimaeric genes derived from genes encoding the chlorophyll *a/b* binding protein^{33,36} were transferred, organ-specifically expressed and shown to maintain their photoregulation upon transfer.

The transferred *lbc3* 5'3'-cat gene is specifically expressed in root nodules of the heterologous *L. corniculatus* host and is regulated at the level of RNA. We therefore infer that the DNA sequences responsible for the nodule-specific expression of the *lbc3* gene are located on the 2-kb 5'-flanking or 0.9-kb 3'-flanking region of the chimaeric *lbc3* 5'3'-cat gene and that interactions at these sequences control the activity of the *lb* promoter. The most reasonable explanation of our results is that the regulation



Fig. 5 (Left) Determination of the transcription initiation site for the *lbc*₃ 5'3'-*cat* gene in *L. corniculatus* root nodules. Total RNA isolated from nodules or leaf+stem was oligo(dT) purified and used in a primer extension assay. A synthetic oligonucleotide 5' CAACGGTGGTATATCCAGTG 3' complementary to nucleotides 15 to 34 of the CAT coding sequence was used as primer. Lane 2 shows the cDNA species resulting from extension of the primer on the CAT transcript present in L6-23 nodules. Lane 3 shows the negative control using leaf+stem mRNA from the L5-9 line and lane 4 the negative control with mRNA from untransformed nodules. No other specific cDNA species were detected. Lanes 1 and 5 show the sequence ladder used to size the cDNA. A cDNA 83 nucleotides long would be expected if the soybean cap site at (+1) was used. The cap sites corresponding to the 85, 86, 87, 89 and 90 nucleotide long cDNA species generated are indicated with asterisks on the partial sequence of the *lbc*₃ 5'3'-*cat* 5' region given below. The translation initiation codon of the CAT coding sequence and the TATA box of the *lbc*₃ promoter are underlined. The primer was end labelled with ³²P using polynucleotide kinase and the extension assay was according to Boel *et al.*⁴⁷.

of the *lb* promoter is exerted on the initiation of transcription, but other models for regulating the level of RNA cannot entirely be ruled out.

Haem, the prosthetic group in globin proteins, controls the efficiency of *lbc*₃ 5'3'-*cat* mRNA translation in yeast³⁷. A similar translational control of leghaemoglobin gene expression might therefore also exist in root nodules, but this has not been investigated here.

Sequences required for expression of photoregulated genes are present in 5' flanking regions^{33,36,38-40}. A short 33-base-pair (bp) sequence located between the TATA box and the transcription start site is sufficient to confer light induction on the ribulose 1,5-biphosphate carboxylase *rbcS-E9* gene³⁹ while the *rbcSss3.6* gene has light-responsive and enhancer-type regulatory sequences upstream of the TATA box⁴¹. Organ specificity and light-induction control regions are both located on a short DNA fragment derived from the pea chlorophyll *a/b* binding protein gene³³. The available evidence, therefore, suggests that DNA sequences controlling organ specificity are closely linked to the promoter. We therefore expect that the regulatory sequences involved in nodule-specific expression are located on the *lbc*₃ 5' flanking region. The correct initiation of transcription and organ-

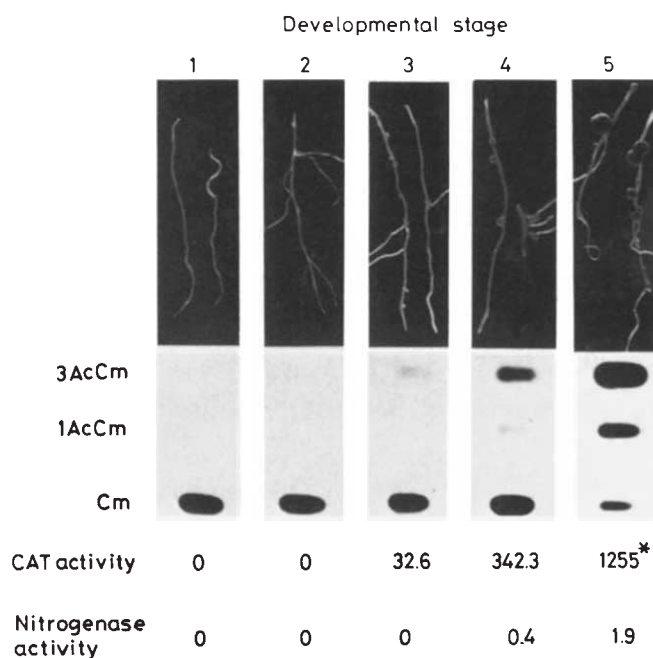


Fig. 6 (Above) Comparison of the *lbc*₃ 5'3'-*cat* gene expression in developing root nodules. CAT and nitrogenase activity were measured on root pieces with nodules in different developmental stages. Stage one shown in panel 1: no visible nodules. Stage 2, panel 2: emerging nodules. Stage 3, panel 3: distinct white nodules. Stage 4, panel 4: small pink nodules. Panel 5 shows nodules in various later states of maturity. The CAT activities and nitrogenase activities follow the developmental control of *lb* gene expression in soybean root nodules. *, This enzymatic activity is only valid for the substrate-limited reaction shown.

Methods. 400 mg root tissue carrying appropriate nodules was analysed for each developmental state. CAT activity was determined in half of this material according to the previously given procedure. The other half was used to measure acetylene reduction capacity of the nodules. Roots and nodules were incubated for 1 h with an atmosphere of 10% acetylene in Suba Seal-stopped 7 ml Bijoux bottles. Ethylene was measured on a gas chromatograph and nitrogenase activity determined as nmol ethylene per μ g protein per h. CAT activity is in c.p.m. per μ g protein per h.

specific expression of the *lbc*₃ promoter in the distantly related legume *L. corniculatus* has further implications concerning the control of nodule expressed genes. *Lotus corniculatus* belongs phylogenetically to the tribe Loteae while soybean belongs to the Phaseoleae⁴². The microsymbiont *Rhizobium loti* is a species separate from the soybean symbiont *B. japonicum*¹ and nodulates plants from a different cross-inoculation group. It appears therefore that both a putative bacterial induction signal and DNA sequences involved in nodule-specific expression of plant 'nodulin' genes are conserved in the legume *Rhizobium* sym-

biosis. Efficient nodulation of different plant species by broad host range isolates of *Rhizobium* and the wide susceptibility of certain host plants, support this idea^{1,43}.

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Fluorescence detection in automated DNA sequence analysis

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We have developed a method for the partial automation of DNA sequence analysis. Fluorescence detection of the DNA fragments is accomplished by means of a fluorophore covalently attached to the oligonucleotide primer used in enzymatic DNA sequence analysis. A different coloured fluorophore is used for each of the reactions specific for the bases A, C, G and T. The reaction mixtures are combined and co-electrophoresed down a single polyacrylamide gel tube, the separated fluorescent bands of DNA are detected near the bottom of the tube, and the sequence information is acquired directly by computer.

THE structural analysis of DNA has an increasingly important role in modern molecular biology. About 4×10^6 bases of DNA have been sequenced since the introduction of the enzymatic method of rapid sequencing developed by Sanger and co-workers^{1,2} and the chemical method developed by Maxam and Gilbert³. Typically, four separate reactions are performed on the particular DNA segment to be analysed. In the enzymatic method, these reactions produce DNA fragments terminating in either adenosine (A), cytosine (C), guanosine (G) or thymidine (T). In the chemical method fragments terminating in G, G+A, C+T, or C are typically produced. In both cases the four sets of reaction products are electrophoresed in adjacent lanes of a high-resolution polyacrylamide gel. An autoradiographic image of the gel is produced which can be examined

to determine the relative lengths of the DNA fragments generated in each of the four reactions. The DNA sequence is inferred directly from this information. Both these techniques are highly effective but are also very labour-intensive, fairly expensive and involve the use of radioisotopes. For these reasons, and because many genes remain to be sequenced (there are 3×10^9 bases in the human genome alone), we have undertaken the development of an automated and non-isotopic method of DNA sequence analysis.

Strategy

Our approach has two major aims. The first is to acquire, store and analyse sequence information directly by computer. Doing this in real-time during the gel electrophoresis eliminates the

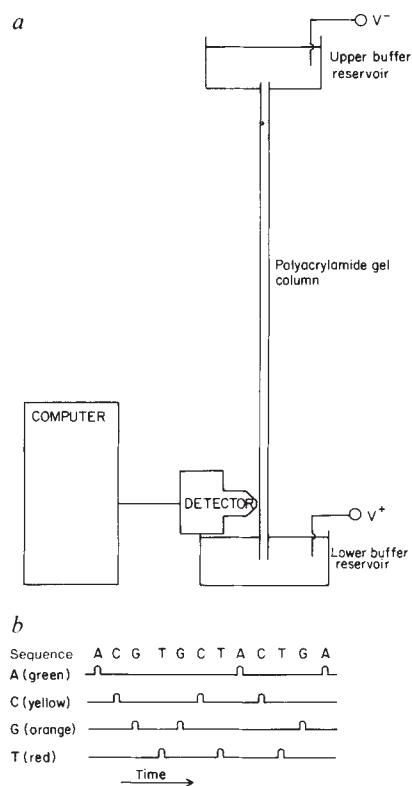


Fig. 1 *a*, Simplified diagram of the automated DNA sequencer. *b*, Idealized output from the automated DNA sequencer.

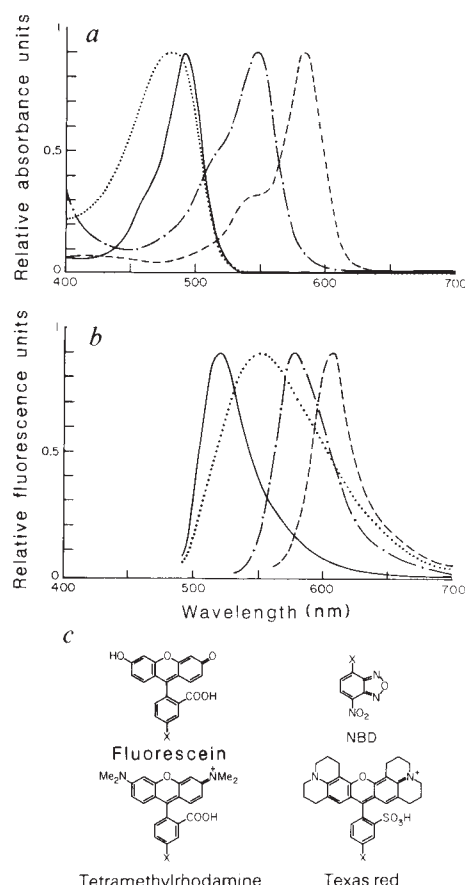


Fig. 2 *a*, Absorption spectra of the four dyes used in the DNA sequencer: —, fluorescein; ·····, NBD; ---, tetramethylrhodamine; - · - ·, Texas Red. *b*, Fluorescence emission spectra of the four dyes; the same line types as in *a*, are used to denote the dyes. *c*, Chemical structures of the four dyes. X, The moiety to which the dye is bound, for example, an oligonucleotide primer. **Methods.** All spectra were obtained in 10 mM sodium carbonate buffer, pH 9.0; absorption spectra were taken on an H/P 8451 spectrophotometer; fluorescence spectra were taken on a Perkin-Elmer MPF4 spectrofluorimeter (uncorrected). The following dye derivatives were used for measurements: fluorescein isothiocyanate (FITC), NBD amino-hexanoic acid, Texas Red (all from Molecular Probes, Junction City, Oregon); and tetramethylrhodamine isothiocyanate (Research Organics, Inc., Cleveland, Ohio).

transfer of information from gel to film (autoradiography), the tedious manual analysis and the need to run several overlapping gels; instead, a single gel can be run for as long as resolvable sequence is obtained. The second aim is to avoid the use of radioisotopes, which are hazardous, costly and unstable.

With these considerations in mind, we have developed a strategy for the automated sequence analysis of DNA which is based on two key points (proposed initially in ref. 4). First, fluorophores are used for detection rather than radioisotopes. Fluorescence provides sufficient sensitivity for real-time optical detection of the small amounts of DNA present in DNA sequencing gels ($\sim 10^{-15}$ mol per band) (see the discussion below). Second, four different fluorophores are used, one for each of the base-specific reactions. The reaction products are combined and co-electrophoresed, and the DNA fragments generated in each reaction are detected near the bottom of the gel and identified by their colour. These fluorescence data are continuously acquired and stored by computer during the electrophoresis. The data may then be analysed by computer to yield the DNA sequence. An idealized version of this process is depicted in Fig. 1.

We applied this strategy to Sanger's enzymatic method of DNA sequence analysis. We use chemically synthesized fluorescent oligonucleotide primers to label the DNA fragments. When these fluorescent primers are used instead of the usual underivatized primer in the enzymatic sequencing reactions, the DNA fragments generated in the reactions are fluorescent.

Chemistry

The first step in implementing this strategy was to develop chemistry for the synthesis of the fluorescent oligonucleotide primers^{4,5}. Briefly, we synthesized a derivative of thymidine which contains a phosphoramidite moiety at the 3' carbon and a protected amino group at the 5' carbon. When this molecule is used in the final addition cycle of oligonucleotide synthesis by the phosphoramidite method (see ref. 6 for review), the product after deprotection and cleavage from the solid phase is an oligonucleotide containing a single aliphatic amino group

at the 5' terminus. This material may then be conjugated readily to any of various commercially available amino-reactive fluorescent dyes to yield the corresponding oligonucleotide derivative.

The selection of a set of four fluorophores was central to development of the DNA sequencer. Several criteria were used in the selection of these fluorophores. (1) The absorption and emission maxima had to be in the visible region of the spectrum, as far to the red end as possible, to minimize scattering and fluorescence background. (2) To be able to distinguish between the dyes effectively, their emission maxima had to be well resolved from one another. (3) The dyes had to be highly fluorescent to provide sufficient detection sensitivity. (4) The dyes should not significantly impair the hybridization of the oligonucleotide primer, as this would also decrease the efficiency of synthesis in the sequencing reactions. Finally, the electrophoretic mobility of the DNA fragments should not be distorted unacceptably by the presence of the dyes. The chemical structures and absorption and emission spectra of the four dyes we have chosen are shown in Fig. 2. Their absorption and emission maxima range from 486 nm (4-chloro-7-nitrobenzo-2-oxa-1-diazole (NBD) absorption maximum) to 604 nm (Texas Red emission maximum), well within the visible region of the spectrum. The emission maxima are evenly spaced at 30-nm

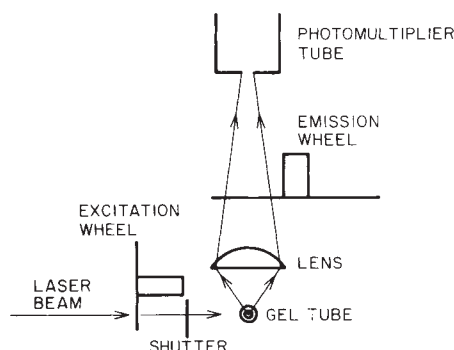


Fig. 3 Diagram of the optical configuration used for within-gel detection of fluorescence at four different wavelengths.

intervals, allowing reasonably good spectral discrimination. All the dyes have good fluorescence sensitivity, and are widely used and readily available. Conjugates of these dyes and an oligonucleotide primer (with the sequence 5' CCCAGTCACGACGTT 3', used in the single-stranded M13 phage vectors) were prepared and tested in conventional sequencing reactions using radioisotope detection (see ref. 4 for details). All the dye-primer conjugates were effective in the DNA sequencing reactions. The dyes do, however, affect the electrophoretic mobility of the DNA fragments to which they are bound; this necessitates a mobility correction to the sequence data which is discussed in the section on data analysis. The suitability of these primers in fluorescence-based detection was tested in instrumentation constructed for that purpose, as described below.

Instrumentation

The primary consideration in the design of the fluorescence detection apparatus was sensitivity. We performed preliminary experiments using radiolabelled nucleoside triphosphates of known specific activity to measure the amount of DNA present in a single band of a conventional sequencing gel, and obtained values of the order of 10^{-15} – 10^{-16} mol DNA per band. If the DNA is assumed to occupy a volume of 1 mm^3 , the DNA concentration is 10^{-9} – 10^{-10} M. Therefore, the instrument had to be capable of detecting dye concentrations of that order. This level of detection is readily achieved in commercial spectrofluorimeter systems. However, detection directly within the gel leads to much higher background scatter from the gel and from the circular glass tubing than is normally encountered, leading to decreased sensitivity. We therefore chose to use a laser excitation source in order to obtain maximum sensitivity, and have continued to use the laser source in subsequent work.

Figure 3 shows a schematic diagram of the optics used for detection of the fluorescent bands of DNA in the tube gel. The beam from an argon-ion laser (Lexel 65-0.1) operated in 'multiline' mode is passed through a 1-nm-bandpass interference filter (to select out either the 488-nm or 514-nm laser line for excitation) mounted on a circular 'excitation' wheel and directed into a gel tube (glass or quartz tubing of 1–2 mm internal diameter; typically a 50-cm-long 8% polyacrylamide/6 M urea gel with the laser beam at 40 cm from the top of the gel) suspended between two tanks containing electrophoresis buffer. A single, fast-collection lens (Melles Griot 01 LAG 013, F/0.7) is used to collect the emitted light and focus it through a 10-nm-bandpass filter (Corion S10-x-R series, where x denotes the bandpass wavelength in nanometres, chosen to match the emission maximum of the dye being used) mounted on a second circular emission wheel, and onto the aperture of a photomultiplier tube (Hamamatsu R928). The optics are mounted on an optical table (MST46 with NN4-28 legs; Newport Research Corporation) enclosed in a dark room which has been painted black, and appropriate baffling is used to prevent stray light from reaching the photomultiplier tube. Four interference filters are mounted

at equally spaced intervals on each filter wheel, and a given excitation filter is always used in conjunction with a given emission filter. The filter pairs used are (in the order excitation filter, emission filter) 488, 520; 488, 550; 514, 580; 514, 610. The use of two different excitation wavelengths gives more efficient excitation of the different dyes (tetramethylrhodamine and Texas Red are excited more efficiently at 514 nm, whereas fluorescein and NBD are excited more efficiently at 488 nm) and thereby provides greater detection sensitivity. Neutral-density filters (Melles Griot BK-7 series) are also used in conjunction with the bandpass excitation filters to balance the baseline gel scatter levels obtained in each filter position. Light-emitting diodes and phototransistors are used to sense the position of the wheels. The excitation and emission wheels are accelerated and decelerated between the different filter positions using time intervals between pulses to the stepper motor that have been chosen to yield a constant acceleration. The movement of the wheel from one position to the next took 0.7 s, and the wheel was held stationary at each position for 0.8 s, giving a total time of 1.5 s between data points. A Uniblitz shutter (Vincent Associates) to protect the photomultiplier tube from excess scattered laser light was inserted into the laser beam and appropriate electronics were constructed to close the shutter if the photomultiplier tube (PMT) current exceeded a pre-set limit. Software was written to close the shutter, align both of the wheels into their starting positions, and then to re-open the shutter and commence data acquisition.

Typically, 8,000 points were taken for each of the four filter positions (total of 32,000 points) in a 13-h run; this rate of data acquisition gives 40 or more data points per DNA peak, under the electrophoretic conditions used. Filtering of the data to eliminate high-frequency noise was performed at three levels. First, an analog filter with a 100-ms time constant was used on the phototube output. Second, at each filter position, 200 data points were taken in succession and averaged, to yield a single point which was stored. At the 40-kHz rate of the Tecmar analog-to-digital converter, this required ~ 5 ms. Finally, the data were smoothed further with a low-pass Fourier filter included in the analysis (see below). Figure 4 shows a set of four-dye data obtained with this system. The raw data are shown in Fig. 4a; b and c show the same data after analysis. The procedures used in data analysis are described briefly below. These data demonstrate the practicality of this four-dye fluorescence approach for automated DNA sequence analysis.

Data analysis

If the raw data obtained with this instrument were as readily interpretable as the idealized data presented in Fig. 1b, very little data analysis would be required to determine the DNA sequence. In reality, however, several factors render the analysis of the data more complex. First, the emission spectra of the different dyes overlap substantially (see Fig. 2b); because of this, peaks corresponding to the presence of a single dye are seen in more than one channel. To discern the DNA sequence, one must therefore perform a multicomponent analysis to determine the different amounts of the four dyes present in the gel at any given time. The second major factor complicating the analysis is that the different dye molecules impart non-identical electrophoretic mobilities to the DNA fragments (see ref. 4). The fluorescein- and rhodamine-labelled DNA fragments move as if they were approximately one base longer than the NBD-labelled fragments, and the fragments labelled with Texas Red move as if they were approximately $1\frac{1}{2}$ bases longer. These mobility shifts must be compensated for. The third complicating factor in the analysis of the data comes from the imperfections of the enzymatic method of DNA sequence analysis itself. It is well known that the enzymatic method suffers from several anomalous traits, such as substantial variations in the intensity of bands in a given reaction, and occasional areas that prove difficult to sequence (thought to be associated with regions of

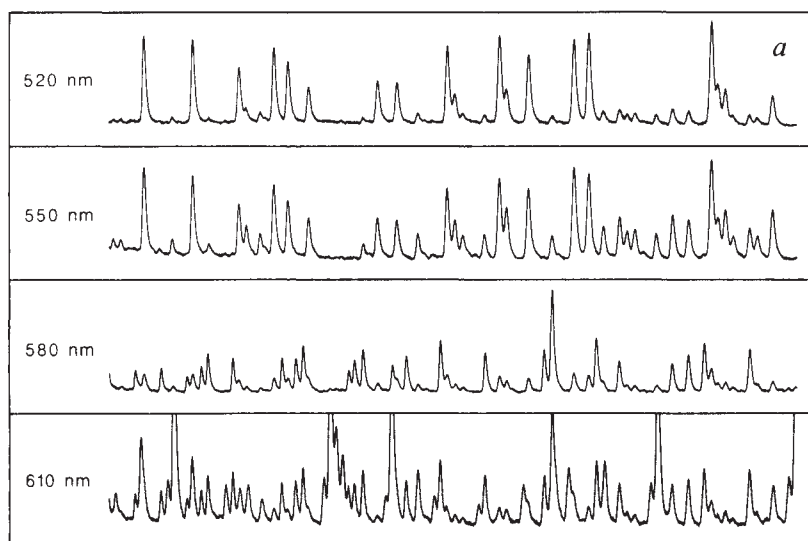
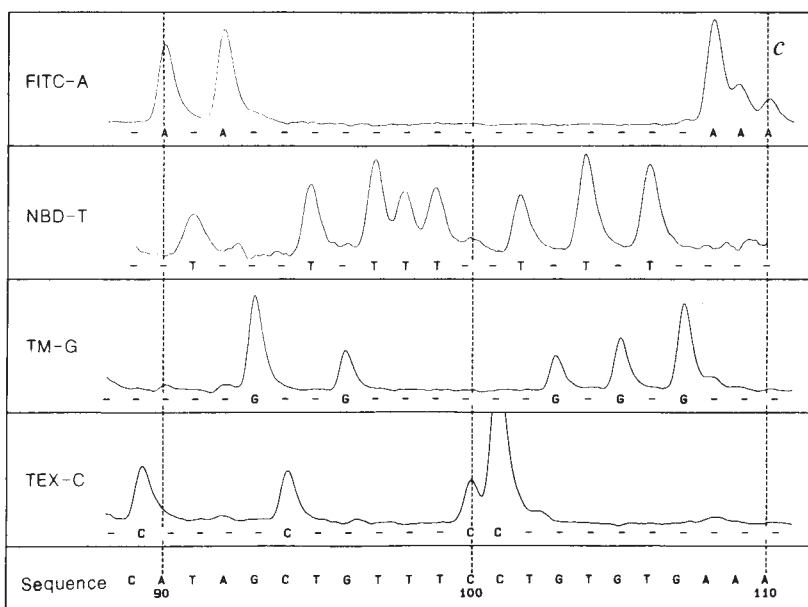
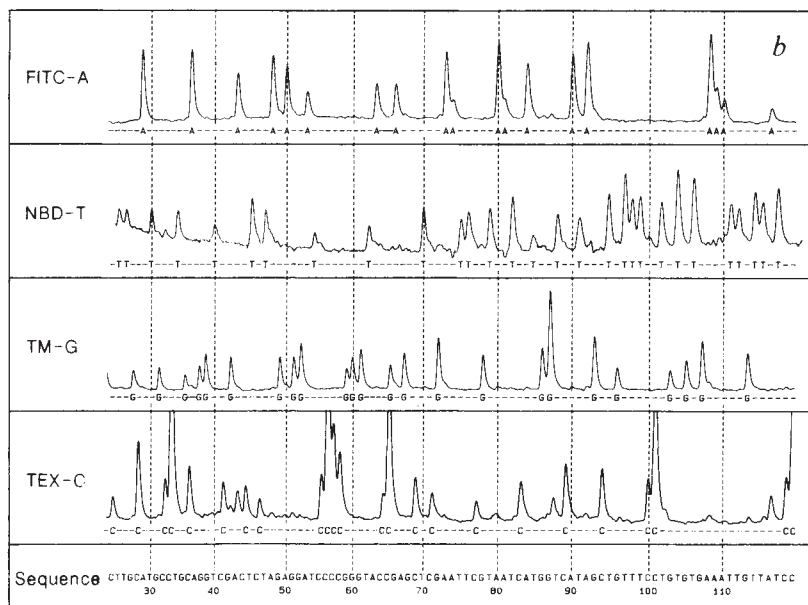


Fig. 4 Fluorescence data (*a*, raw; *b*, analysed) obtained from a mixture of four sequencing reactions. *c*, Expanded plot of 1,000 points of the analysed data. The mixture was applied to a single gel tube (quartz tubing with i.d. 1.3 mm; Heraeus Amersil, Sayreville, New Jersey) and detected using the optical configuration shown in Fig. 3 and described in the text. The relative amounts of each reaction used were chosen to give comparable signal intensities for each dye. This mixture contained 0.025 units of an FITC-A reaction, 0.1 units of Texas Red-C (Tex-C), 0.15 units of tetramethylrhodamine-G (TM-G), and 0.4 units of NBD-T, mixed with one volume of formamide, in a total volume of 14 μ l. A unit amount of sequencing reaction contains 0.4 pmol of template DNA and 0.8 pmol of primer DNA. The protocols used in the preparation and loading of the sequencing reactions are given in detail in ref. 6. See text for details of the data analysis.



secondary structure in the DNA itself). These complications must be handled in the analysis.

The procedures we have used in data analysis are described in detail in ref. 5. Briefly, the analysis consists of five steps: (1) High-frequency noise is removed by using a low-pass Fourier filter. (2) The time delay (1.5–4.5 s) between measurements at different wavelengths is partially corrected for by linear interpolation between successive measurements. (3) A multicomponent analysis is performed on each set of four data points; this computation yields the amount of each of the four dyes present in the detector as a function of time. (4) The peaks present in the data are located. (5) The mobility shift introduced by the dyes is corrected for using empirically determined correction factors. Figure 4b shows the processed data obtained when these five steps were applied to the raw data of Fig. 4a, and Fig. 4c shows an expanded plot of a region of 1,000 data points taken from Fig. 4b; the DNA sequence is aligned with the data. The correspondence between the sequence and the order of peaks obtained in the different channels is evident.

To date we have successfully executed hundreds of single-dye runs, 20–30 two-dye runs, and about 10 four-dye runs. In single-dye runs we often obtain well resolved and clearly discernible sequence beyond base 400 in ~16 h (data not shown). In four-dye runs we generally have obtained ~200 bases of clear sequence information. Present efforts are directed towards further defining and optimizing the parameters important in obtaining good resolution.

Sensitivity

The question is often raised of how the sensitivity of the fluorescence method compares with the sensitivity of conventional radioactive methods. The comparison is complicated by the fact that in the radioactive detection methods, the signal is integrated (by exposure on film) over long time periods ranging from hours to days. In contrast, the fluorescence approach uses real-time acquisition with integration times of a fraction of a second per data point; sensitivity is naturally much lower when the signal is integrated over such relatively short times. If one ignores this aspect, and simply considers sensitivity to be a measure of the amount of material one can detect over background in either method, the radioactive method clearly has higher sensitivity. The most sensitive of the four dyes we are using is fluorescein, and when small amounts of fluorescein-labelled primers are detected during electrophoresis on short tube gels, we obtain a minimum detectable level (defined here as a signal-to-noise ratio of 1) of ~10 amol (1 amol is 10^{-18} mol). Approximate minimum detectable levels obtained for the other dyes are NBD, 40 amol; tetramethylrhodamine, 20 amol; and Texas Red, 50 amol. These levels are about one order of magnitude less sensitive than those typically obtained with autoradiography⁷. The sensitivity of this method could be increased in several ways (see discussion below), which would be of value for extending the amount of sequence information obtained in a run and increasing the accuracy of the analysis.

Future directions

Here we have demonstrated the practicality of a novel method for the automated non-isotopic sequence analysis of DNA. This work opens up several avenues for further investigation. First, the fluorescence sequencing method that we have described will benefit from optimization of several interacting factors, including (1) the gel itself, including its length, diameter and composition (for example, different acrylamide percentages, gradients), (2) the procedures and reagents used in the sequencing reactions, (3) the electrophoresis conditions (for example, voltage, current and temperature), (4) the optics and electronics used in data acquisition, (5) the chemistry used in fluorescent primer synthesis (for example, attaching multiple dye molecules per primer to increase sensitivity, adding linker arms to the dyes to compensate for their non-identical electrophoretic mobilities, finding

new dyes with improved fluorescence characteristics), and (6) the software used in data reduction. We have obtained resolved peaks with good signal-to-noise 400 bases into the sequence in some instances, even with the present relatively unoptimized configuration. It is therefore reasonable to expect that an optimized system would yield sequence information beyond position 500 on a routine basis.

The data analysis methods we have developed to date reduce the raw data into a form which is interpretable by the user. We are presently developing the software to perform the final step of data analysis, the interpretative step of assigning the DNA sequence to the data. This interpretation must take into account the high degree of variability in peak intensity obtained in the enzymatic sequencing reactions (see Fig. 4b, particularly the Texas Red-C (Tex-C) reaction), and the possibility of 'false' peaks (see, for example, the false C peaks at base numbers 43, 87 and 116 in Fig. 4b). The software will be able to minimize, but not to eliminate, these problems. It is therefore important that bases which cannot be assigned unambiguously are left unidentified, and that the sequence of unknown DNAs is determined on both strands. A reasonable goal for the system accuracy is a 1% error rate for a single strand, which would give a 0.01% error rate or 1 base in 10,000 for the sequence determined from both strands. This level of accuracy is satisfactory for most applications, and is comparable to or better than the accuracies generally obtained in DNA sequence analysis by conventional methods.

It will also be of great value to apply this fluorescence technology to the chemical method of DNA sequence analysis. This is important for several reasons. First, as mentioned previously, there are occasionally regions of DNA that are difficult to sequence by the enzymatic method but which are usually amenable to analysis by the chemical method. Second, whereas the enzymatic method is characterized by significant peak-to-peak variations in intensity, visible in both autoradiograms and in the fluorescence data presented here, the chemical method generates a much more uniform distribution of fragments of different lengths, which would facilitate data analysis. Third, the simple and inexpensive reagents used in the chemical method can be stored easily, and are easier to handle in a machine for automating the sequencing reactions than the delicate reagents (for example, the Klenow fragment of DNA polymerase) used in the enzymatic reactions. Recent advances in solid-phase methods of sequence analysis by the chemical method^{8,9} will also be of great value in automating those reactions.

Thus, the practicality of a novel non-isotopic method for automated DNA sequence analysis has been demonstrated. It is our hope that this work will help to eliminate much of the labour associated with the structural analysis of DNA, and thereby allow investigators to focus on the more interesting and important questions of modern biology.

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LETTERS TO NATURE

Coordinated Exosat and spectroscopic observations of flare stars and coronal heating

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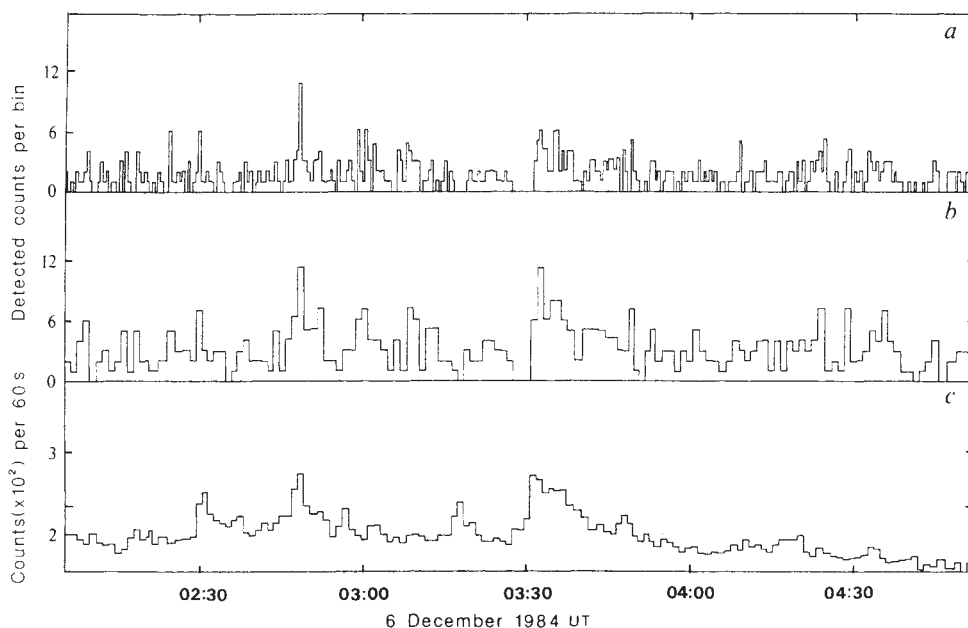
The X-ray flux of dMe stars is thought to arise from two distinct mechanisms, one involving a continuous 'quiescent' emission from a high-temperature plasma and the other involving the dramatic flare events which have long been known to occur on these stars. We present here some results of simultaneous monitoring of the two flare stars, UV Ceti and EQ Peg, with Exosat and ground-based optical spectroscopy. We observe short-timescale variability in the 0.1-2-keV emission from both these objects and, in the case of UV Ceti, find a strong correlation between the soft X-ray and H γ fluctuations. The implication is that much of the low-level X-ray flux previously considered 'quiescent' probably originates from small flare events.

Our understanding of coronal structure and of mechanisms for coronal heating in the Sun and stars has evolved considerably as a result of the solar observations of Skylab and the Solar Maximum Mission (SMM) and the stellar observations of the Einstein Observatory and now Exosat. The Skylab photographs of the Sun showed that the soft X-ray emission originated almost exclusively in active-region loops, in larger loops connecting active regions and in X-ray bright points, which are thought to be unresolved, small-scale loops of newly emerging magnetic flux. In place of the diffuse Parker-type hot corona/wind, the

closed magnetic loop is now widely thought of as the basic unit of coronal structure¹⁻³. By contrast, regions of open magnetic field, called coronal holes, are dark in X rays and appear to be the primary source of the solar wind. Because many of the Skylab and other images emphasize the higher-temperature and more dense structures (the emission for a collisionally excited line is $\sim n(e)^2$), it may well be that there is a significant diffused coronal component, in addition to coronal loops and coronal holes. Whether or not this is the case, the concept of a 'quiet corona' (be it diffuse or the average state of the quasi-steady loop ensemble) has persisted since the discovery of the solar corona. This concept has recently been extended to 'quiet stellar coronae'⁴, the dichotomy being one between the quasi-steady evolution of high-temperature, X-ray-emitting structures, on timescales of hours to days to weeks, and the more obvious variability associated with flares. The heating of the 'quiet corona' and the nature and energetics of solar flares have thus historically been viewed as different and distinct problems.

However, recently, using balloon-borne instrumentation, a significant number of solar hard X-ray microflares have been observed⁵, presumably produced by bremsstrahlung of non-thermal electron beams in the solar chromosphere and transition region. It was found that the average rate of energy deposition above 20 keV and above the detection threshold amounted to $\sim 10^{24}$ erg s⁻¹. In addition, the integral number of events appeared to vary as the inverse of the peak flux down to their detection threshold, in which case the total rate of energy release, allowing for low-level events, could begin to approach the total quiescent solar coronal X-ray luminosity⁶, $L_x = 5 \times 10^{27}$ erg s⁻¹. The timescales for these microflares ranged from seconds to tens of seconds in duration. Elsewhere in the spectrum, short-lived extreme-ultraviolet bursts of subflare magnitude have been observed on the Sun in several transition-region lines; these bursts probably result from the same electron beaming event that creates the hard X-rays⁷. From a re-analysis of Skylab data⁸, frequent subflare-like extreme-ultraviolet brightenings have been found near the foot-points of loops, and using the SMM

Fig. 1 Soft X-ray flux detected by the CMA on Exosat from UV Ceti, compared with the H γ peak intensity from the ESO 3.6-m telescope. *a*, X-rays (LE), bin size 30 s; *b*, X-rays (LE), bin size 60 s; *c*, H γ peak, exposure 60 s.



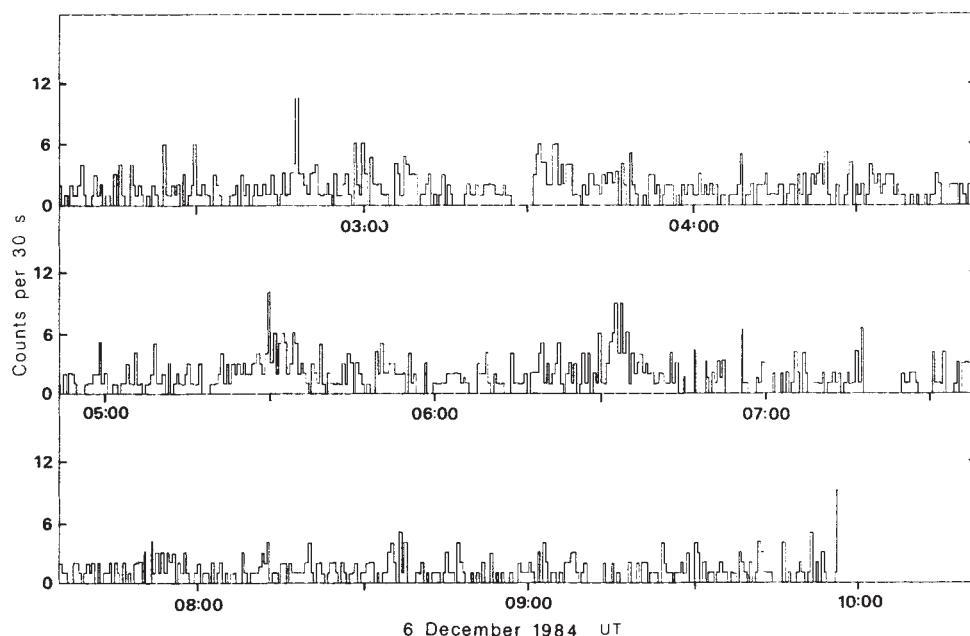


Fig. 2 Soft X-ray flux from UV Ceti for the entire Exosat exposure. The background level is 0.21 counts per 30 s over the source area.

ultraviolet spectrometer polarimeter, almost continual fluctuation has been seen in the Si IV 1,402.8 Å ($T \approx 60,000$ K) and O IV 1401.2 Å ($T \approx 160,000$ K) lines in a solar active region^{9,10}. The timescales for these changes are 10–60 s and the brightenings are sometimes more than a factor of two. The timing of these events is such that there must be an almost instantaneous local heating event followed by radiative cooling. In some cases, a secondary brightening is observed after an impulsive burst, which may be the small-scale analogue of the thermal phase of a flare. More importantly, it has been shown¹¹ that the incidence of extreme-ultraviolet bursts exceeds that of flares by more than two orders of magnitude, which suggests that, in total, they may be as important energetically as flares. These results, together with our own, suggest that heating due to magnetic field reconnection within an active region is proceeding almost stochastically.

The recent studies of correlations between 'quietest' X-ray fluxes and stellar U-band flare energy rates for dMe stars are of particular interest as they point to a possible equality between total energy released in flaring and the energy requirements of the quiet stellar corona. The relationships between the time-averaged U-band flare luminosity, $\langle L_{\text{flare}}; \text{U-band} \rangle$, and coronal X-ray luminosity, L_x (refs 12, 13), are linear over several orders of magnitude, and imply that $L_x / \langle L_{\text{flare}}; \text{U-band} \rangle \approx 10$ –25. This result appears to hold not only for the dMe stars, from which it was established, but also for non-emission M dwarfs, and possibly even for the Sun¹⁴. Because the U-band flare (which is the band most easily measured by optical observers) encompasses only a fraction of the flare energy, it is possible that there is an approximate equality between total flare emission and quiescent coronal emission. However, the mean U-band flare energy is dominated by the relatively large and infrequent flares (>1 per 10 h), and if they are to be the source of coronal heating, it is difficult to see how their energy, once it has been released in a flare, could be stored for times of this order. The present new data demonstrate the importance of continual microflaring on the dMe flare stars.

As part of a coordinated campaign of multi-band observations of stellar flares¹⁵, we observed UV Ceti and EQ Peg on 6 and 7 December 1984, respectively, with ground-based optical telescopes and with the two satellites IUE (International Ultraviolet Explorer) and Exosat. Included among several optical telescopes was the 3.6-m of the European Southern Observatory (ESO), with which intermediate dispersion spectroscopy of the

region 3,600–4,400 Å was obtained using the image dissector scanner on the Boller and Chivens spectrograph. Exposures were of 60 s duration with a short delay of ~10 s between spectra. Although these data have not yet been fully reduced, it was possible to determine, in real time, an uncalibrated peak flux value at the wavelength of H γ . The variation of this peak value will be predominantly due to the H γ emission line itself; however, some continuum variations will also be included.

Because of Exosat pointing restrictions, it is difficult to obtain, from one site, more than ~5–6 h of simultaneous ground-based coverage. In the case of UV Ceti, Exosat observations were made from 02:00 to 10:45 UT on 6 December 1984, but only the first 3 h could be covered from ESO.

The X-ray (0.1–2 keV) photon counts detected by the channel multiplier array (CMA) of the Exosat low-energy imaging telescope (LE) (using the 4,000-Å-thickness Lexan filter) have been analysed using the reduction package at Mullard Space Science Laboratory, University College London. Some gaps are present in the data where data transmission, or small solar flares, have caused the exposure time to fall below 80% of the nominal value. Although small solar flares do not noticeably increase the flux over the area of the CMA detector containing the stellar image, the increase over the entire image area can be sufficient to overload the detector system and cause a reduction in the effective exposure time.

In Fig. 1a, b the X-ray data have been grouped into 30-s and 60-s bins, respectively, and Fig. 1c shows the time variation of the peak H γ flux. The data in Fig. 1a are binned in successive 30-s intervals; however, to allow a better comparison with the H γ peak flux, the 60-s X-ray data (Fig. 1b) have been grouped into 60-s bins that do not follow on from each other immediately, but rather are as nearly as possible coincident with the windows of the H γ observations. Therefore, the total counts in the two X-ray plots will not be exactly the same.

Figure 1 shows two important features. First, the X-ray emission appears to be quite non-uniform, with frequent rises of a factor of two or three in flux, lasting for periods of a few tens of seconds to several minutes. Second, five or six of the X-ray events correspond to similar rises in H γ peak flux. We have investigated this correlation statistically and find that the correlation coefficient between the 60-s data points used to construct Fig. 1b and c is 0.51, or a Student's t value of 7.0. For 143 data points this implies a highly significant correlation between the Exosat and H γ data, with a probability of

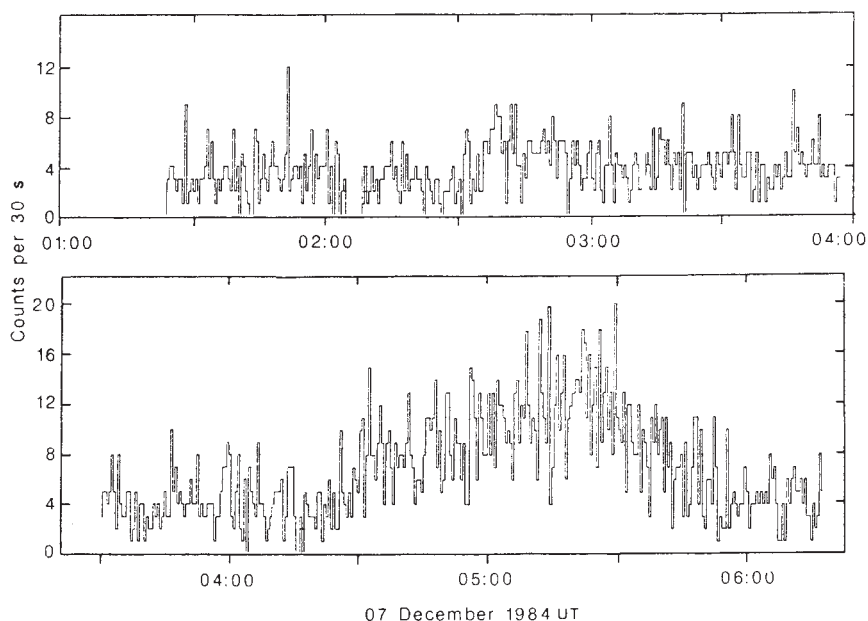


Fig. 3 Soft X-ray flux from EQ Peg. Note the large flare beginning at 04:30 UT. The background level is 0.23 counts per 30 s over the source area.

occurrence by chance of less than 1 in 10^5 .

We conclude that the events seen in the X-ray and H γ fluxes are in fact small flares, which, like flares on the Sun, show increased X-ray emission accompanied by Balmer-line enhancements; the X-rays are presumably due to thermal emission of the flare-heated plasma in one or more loops and the Balmer-line enhancements to chromospheric ablation¹⁶. While both the soft X-ray events and the Balmer-line enhancements on the Sun last rather longer than these events on UV Ceti, the energies are similar: at a distance of 2.62 pc (ref. 17), the energies in the UV Ceti low-energy X-ray events vary from ~ 0.5 to 5×10^{30} erg, similar to the energies of compact solar flares.

Figure 2 shows all of the Exosat data for UV Ceti in 30-s bins. Here again we see evidence for an almost continuously variable soft X-ray flux, and to determine whether this variability is statistically significant, we have binned the data with a bin size varying from 10 to 160 s, and compared the observed number of bins with a given population to a Poisson distribution using a χ^2 test. In all cases, the probability of the observed distribution arising from a random arrival of photons from a constant source is $\leq 0.5\%$. The departure from a Poissonian distribution is in the same sense for each trial bin-size, with both more bins with zero or a low number of photons than expected, and more bins with high numbers of photons than expected. The number of bins found with intermediate numbers of photons is correspondingly decreased. This type of distribution could arise from the clumping together of photons from a source which is variable on timescales of 10–160 s.

In Fig. 3, which shows the soft X-ray flux from EQ Peg on 7 December 1984, we again see evidence of continuously variable emission, followed by a substantial LE (soft X-ray) flare, which was also detected by the ME (medium energy) experiment. The flare lasted for ~ 90 min, with a gradual rise and fall in LE and a more sudden rise in ME. A preliminary analysis of the integrated ME spectral data for the event gave, for effectively zero column density of hydrogen, a best-fit temperature of $\sim 2.2 \times 10^7$ K: this is a typical temperature for the non-impulsive, thermal phase of solar and stellar flares^{18,19}. If we adopt the standard calibration of the Exosat detectors²⁰, and a distance for EQ Peg of 6.41 pc (ref. 17), the total integrated X-ray energy of the flare on EQ Peg is 6×10^{32} erg in LE (0.1–2 keV). This corresponds to the energy of a very large two-ribbon solar flare²¹.

Our data for UV Ceti and EQ Peg suggest that the soft X-ray emission of these stars is almost continuously variable, with

many surges or microflares lasting from tens of seconds to several minutes. These microflares, which have energies of $\sim 2 \times 10^{30}$ erg in the 0.1–2 keV range, are similar to compact solar flares, but possibly more short-lived. The fact that these microflares occur so frequently in the two dMe stars studied indicates that the concept of a 'quiescent' corona for these stars may be erroneous and that we are seeing, not a quiet, uneventful, low-level emission, but a continuum of flares with energies similar to those of compact solar flares. Most probably, if our detection threshold could be significantly reduced, we could see many even smaller X-ray events. The logical consequence of these results is that what we call stellar coronal emission (the X-ray flux) may be just a succession of microflares.

There are now three other lines of evidence pointing to the possibility that coronal heating in dwarf M flare stars is a direct result of energy input through widespread, low-level microflaring^{22,23}: (1) the recently found correlation between time-averaged flare energy release and coronal X-ray luminosity^{12,13}; (2) the high-temperature component identified in the two Einstein Observatory solid state spectrometer observations of 'quiescent' coronal emission from the dMe stars^{24,25} and in many of the Einstein imaging proportional counter pulse-height spectra of dMe stars which have now been reprocessed (J. Bookbinder, L. Golub, R. Rosner and J. H. M. M. Schmitt, in preparation); and (3) the observed variability on timescales of hundreds to thousands of seconds of practically all reprocessed Einstein coronal X-ray fluxes for dKe and dMe stars (C. Ambruster, S. Sciortino and L. Golub, in preparation).

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Metallic ions in cometary comae and plasma tails

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Ion composition measurements made in the environment of the comet Giacobini-Zinner by the International Cometary Explorer (ICE) spacecraft have shown that H_2O^+ is a major ion species, together with CO^+ ions¹. The presence of these two cometary ions has long been evident in optical spectra of cometary ion tails². However, the ICE observations were surprising in that they detected ions of mass 23–24 AMU with a relatively high abundance¹. According to the experimenters, these ions may be either Na^+ or C_2^+ , if not both. We suggest here that the ions detected may indeed be in part Na^+ and/or Mg^+ and that these and other metallic ions (especially Si^+ and Fe^+) may be an important component of the cometary ionosphere and central plasma tail. Our reasons are similar, in principle, to those which account for the prevalence of such ions in sporadic E layers in the terrestrial ionosphere, notably the comparatively short timescales for ionization of their neutral parent atoms and the large difference between the rates of dissociative and radiative recombination^{3,4}.

Because of its strong optical emission, the source strength of C_2 relative to that of H_2O and the scale length for photodissociation and/or photoionization have been well determined. The average values are: $Q(\text{C}_2) \sim 4 \times 10^{-3} Q(\text{H}_2\text{O})$ and $R(\text{C}_2) \sim 1.2 \times 10^5 \text{ km}$, respectively⁵. In comparison, the corresponding scale length for the ionization of the water-group neutrals (that is, H_2O , OH and O) is a factor of 10 larger. It is possible, therefore, that the faster ionization of C_2 radicals in a more limited region could lead to some enhancement of the relative abundance of C_2^+ ions with respect to H_2O^+ ions in a region $< 10^5 \text{ km}$ from the tail axis.

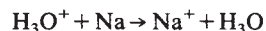
Sodium D-line emissions and emissions from other metallic species (Ca, Cr, Co, Mn, Fe, Ni, Cu, V, Si and K) are usually seen most prominently in Sun-grazing comets or bright comets at solar distances $< 0.5 \text{ AU}$ (refs 6, 7). It is often argued that these emissions originate from atoms released by the sublimation of refractory dust grains⁸. At a solar distance of 1 AU, however, the sublimation rate of such dust grains is negligible and essentially no metal atoms should be released. Sodium D-line emissions were detected in comet West (1975n) when it was at a solar distance of 1.4 AU, which gave rise to the suggestion that a component of Na atoms could be embedded in volatile icy grains or matrix in atomic or molecular forms such as NaOH (ref. 9). Although estimates for the production rate of these Na

atoms (or their parent molecules) are very uncertain (an upper limit of $Q(\text{Na})/Q(\text{H}_2\text{O}) \leq 10^{-3}$ may be set), such observations, together with the ICE ion composition measurement, call attention to the potential importance of Na^+ ions and metallic ions, in general, in cometary comae and ionospheres. Below, we discuss several implications of metallic ion production in the coma environment and, in particular, emphasize that metallic ions could be a significant component of the central ionosphere and ion tail if their production rates are sufficiently large. The relative (solar) abundances of the most important species concerned are Na (0.06), Mg (1.00), Al (0.08), Si (0.95), Ca (0.07) and Fe (0.85)¹⁰.

Note that, in addition to sublimation from non-volatile dust grains and possibly from volatile material containing their parent molecules, sputtering of dust grains and the nucleus itself by solar-wind protons and the energetic 'pick-up' ion created in the coma should provide a source of secondary neutral atoms ($\sim 90\%$) and ions ($\sim 10\%$)¹¹. For a comet with a gas production rate up to that of Halley ($Q = 10^{30}$ molecules s^{-1} at 1 AU) and a dust production rate $\dot{m}_d/\dot{m}_g \sim 1$, the effective cross-sectional area of the dust coma is estimated to be 10^{17} cm^2 assuming a cutoff radius of $1 \mu\text{m}$ (ref. 12). With a solar-wind flux of $3 \times 10^8 \text{ cm}^{-2} \text{ s}^{-1}$ and a sputtering yield of about unity, the effective sputtering rate of neutral atoms and ions from the dust coma is of the order of $5 \times 10^{24} \text{ s}^{-1}$, which is a factor of 2×10^5 less than the volatile gas production rate. This alone might not be sufficient to account for the relatively high fluxes of ions of mass 23–24 detected by the ICE spacecraft at $2 \times 10^4 \text{ km}$ from the tail axis. Nevertheless, secondary ions must be produced by sputtering in the coma and their fluxes may be detectable; there are processes which in any case tend to enhance their fluxes relative to those of molecular ions. Furthermore, the local production rates of sputtered atoms and ions may be larger than we have estimated here if $m_d \gg m_g$ locally (as a consequence of differing expansion speeds of the gas and dust components) and if there is a significant component of dust particles with radii $< 1 \mu\text{m}$.

In general, the solar-wind plasma should not be able to reach the surface of the nucleus but the large gyroradii ($\sim 1\text{--}5 \times 10^4 \text{ km}$) of energetic pick-up ions may allow them to do so. Thus the nucleus and inner coma should be continuously subject to sputtering even when the outgassing rate is large. However, because of the small area of the nucleus ($\leq 10^{12} \text{ cm}^2$), the production rate of neutral atoms and ions arising from direct energetic particle sputtering is many orders of magnitude less than the sublimation rate of H_2O and other gases. Indeed, only when a comet nucleus is inactive at large solar distances would surface sputtering have a major role in releasing surface material into the surrounding coma region^{13,14}.

Despite the relatively low production rates of sputtered atoms, it is important to recognize that by virtue of their short photoionization timescales ($\sim 3 \times 10^4 \text{ s}$ at 1 AU (ref. 9) compared with $3 \times 10^6 \text{ s}$ for H_2O), together with the fact that a substantial fraction of the sputtered gas is already ionized, metallic ions could become relatively enriched. The very fact that such neutral atomic species as Na appear in the spectra of some comets suggests that their photoions should be abundant. The ionization potentials of these atoms are such that they are photoionized by hydrogen Lyman- α radiation, whereas the more abundant molecules require photons of 12 eV or more for photoionization. In addition to photoionization, metallic ions can be produced by charge transfer reactions such as



which has a rate coefficient $\leq 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ (ref. 15); this process may be important in the denser regions of the cometary ionosphere where the H_3O^+ density is $\sim 10^4 \text{ cm}^{-3}$.

It is difficult to find reactions which can neutralize metallic ions faster than radiative recombination, which with a typical recombination coefficient of $10^{-12} \text{ cm}^3 \text{ s}^{-1}$ is itself unimportant in the present context. To remove metallic ions quickly they

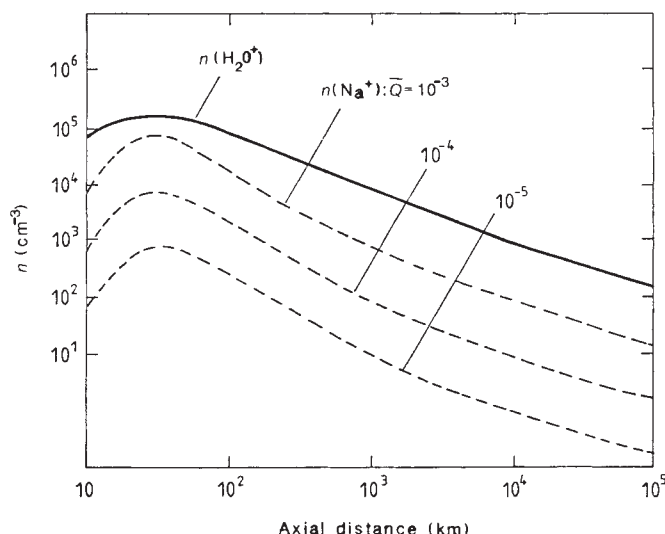


Fig. 1 Comparison of the number density of the $\text{H}_2\text{O}^+/\text{H}_3\text{O}^+$ ions with that of Na^+ (or other metallic ions) for different relative source abundances of the metallic neutrals, $\bar{Q} = Q(\text{Na})/Q(\text{H}_2\text{O})$. In the calculation, simulating the free-streaming ionospheric flow along the tail, the flow speed increases linearly from 1 km s^{-1} near the nucleus to 100 km s^{-1} at $3 \times 10^4 \text{ km}$ from the nucleus. The metal ion enhancement in this case is entirely the result of the relatively high ionization rate of their atoms in comparison with that of water molecules.

must first be converted to some form of molecular ion so that dissociative recombination is a possibility. However, the rates of formation of such molecular ions are low, except perhaps very close to the nucleus: for example, in the case of Na^+ the process



has a rate coefficient of only $1.6\text{--}6.1 \times 10^{-16} \text{ cm}^3 \text{ s}^{-1}$ at 20 K (ref. 16). This is competitive with radiative recombination in regions where the H_2O density is 10^4 or more (that is, within $3 \times 10^4 \text{ km}$) but provides loss rates for Na^+ ions which are significantly only in regions where the H_2O density exceeds 10^9 cm^{-3} and the electron density is $\geq 10^2 \text{ cm}^{-3}$.

In the inner coma, a basic (convective) timescale is determined by the plasma flow characteristics. These are, in turn, controlled by drag which resists motions of plasma relative to the neutrals and by the tension stress created by strongly curved magnetic field lines which pull the plasma inwards towards the nucleus on the upstream side and eventually out along the tail on the downstream side¹⁷. The flow speeds in the 'ionospheric' region surrounding the nucleus may be quite small, perhaps $10^2\text{--}10^3 \text{ m s}^{-1}$, corresponding to convective timescales of $10^4\text{--}10^5 \text{ s}$. The tail plasma flow behind the nucleus can be regarded to a first approximation as an expansion of the ionospheric plasma along a quasi one-dimensional tunnel, accelerated by the thermal pressure gradient and magnetic field streams into a region of negligibly small neutral drag. In this manner, flow speeds of $10\text{--}30 \text{ km s}^{-1}$ may be achieved in $\sim 3 \times 10^4 \text{ km}$, giving a convective timescale of $\sim 10^3\text{--}10^4 \text{ s}$. Detailed calculations are required to analyse these processes fully, involving the ion chemistry as well as the plasma flow dynamics, but the essential features can be demonstrated by a simple approach.

Figure 1 shows results from a model calculation with a range of the relative production rate of the metallic atoms ($Q(\text{Na})/Q(\text{H}_2\text{O}) = 10^{-5}, 10^{-4}$ and 10^{-3}). Ionospheric plasma flow in the tailward direction is simulated here by converting to a one-dimensional geometry with the flow acceleration approximated by assuming $d(\log A)/dx = (2/x) \exp(-x/X_a)$ for the stream-tube area A and an acceleration $dV/dx = (100 \text{ km s}^{-1})/X_b$. The scale lengths are taken to be $X_a = 3 \times$

10^3 km and $X_b = 3 \times 10^4 \text{ km}$. This calculation demonstrates that an enhancement of a factor of ~ 100 could be introduced into the metal ion abundance simply as a result of more rapid ionization.

There is a second important effect which could enrich the metallic ion component of the cometary plasma: in the sunward region of the ionosphere, which is a region of very low speed flow, metallic ions carried in or produced *in situ* could gradually replace H_2O^+ as the dominant ion as a consequence of their differing rates of recombination (see Fig. 2). The lifetime of ions against recombination is $\tau \sim 1/\alpha N_e$, where α is the recombination coefficient and N_e the electron density. For metallic and other atomic ions the (radiative) recombination coefficients are typically of the order of $10^{-12} \text{ cm}^3 \text{ s}^{-1}$, whereas for molecular ions, which undergo dissociative recombination, $\alpha \sim 10^{-6} \text{ cm}^3 \text{ s}^{-1}$. According to magnetohydrodynamic (MHD) simulations (J. A. Fedder, personal communication), ions produced in or brought into the central coma reside for as long as 10^5 s with electron densities of $10^2\text{--}10^4$ or more; in these circumstances the metallic ions simply accumulate ($\tau \sim 10^8\text{--}10^{10} \text{ s}$), whereas molecular ions continually recombine ($\tau \leq 10^2\text{--}10^4 \text{ s}$). Thus, as plasma is carried through the coma by contraction of the looped magnetic field lines to eventually form the plasma tail of the comet, it should become progressively enriched in atomic, especially metallic, ions to a much greater extent than would be implied by their higher ionization rates alone. The formation of such a metallic ion-rich zone by 'magnetic scavenging' in the neutral atmosphere of a comet is analogous to the occurrence of sporadic E in the Earth's ionosphere, where, following rapid photoionization of metallic atoms, neutral atmospheric wind shears in the presence of the geomagnetic field produce the scavenging effect. This, in turn, leads to the formation of metallic ion-rich layers of high plasma density in which the molecular ions may actually be depleted relative to the surroundings^{3,4,18,19}.

The ICE spacecraft passed through the tail of comet Giacobini-Zinner along a path very favourable for the detection of metallic ions but unfortunately it was not designed for this purpose and so only a hint of their presence may have been obtained. However, the Giotto spacecraft has five mass spectrometers of various types which may be able to detect and identify metallic ions. Such ions should be most prevalent in the region immediately surrounding the nucleus ($<10^4 \text{ km}$) but

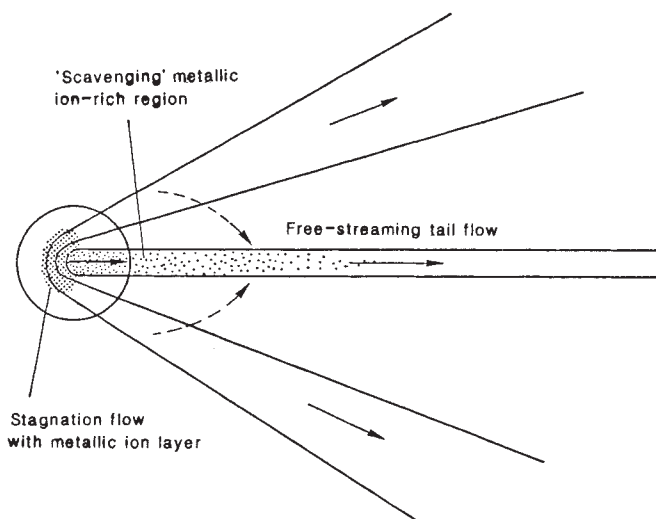


Fig. 2 A schematic view of how the metallic ions might be accumulated in the low-speed flow region of the cometary ionosphere. Metallic ions produced in the coma upstream from the nucleus are carried inwards and concentrated, without suffering recombination losses as do molecular ions, before eventually escaping downstream along the tail.

some should be detectable at larger distances. In some cases, the metallic ions may be submerged beneath large peaks associated with more abundant ions but Fe^+ , in particular, may be relatively free of such interference and hence be more easily identifiable.

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Gas-like nature of the sodium anion in solution

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During the past decade a considerable amount of evidence has accumulated for the existence of an anion of sodium, Na^- , as a stable, long-lived entity in certain non-aqueous solvents¹⁻⁷. The term sodium anion refers to a spherically symmetric diamagnetic species in which, in the orbital model of atomic structure, the two valence electrons are accommodated in the 3s orbital of sodium⁷. We report here direct measurements of ^{23}Na nuclear spin-lattice relaxation rates for Na^- , coupled with nuclear magnetic resonance (NMR) chemical shift data, which are used to show that Na^- in solution is almost completely decoupled from its environment, behaving as if it were in the gas phase.

The systems chosen for study were solutions of sodium and mixed sodium/alkali metal samples (Na/M , where $\text{M} = \text{K}, \text{Rb}$ and Cs) in the solvent 1, 4, 7, 10-tetraoxacyclododecane (12-crown-4, 12C4; Fig. 1). This solvent has a powerful and specific ability to complex alkali cations. For example, the dissociation of sodium/potassium alloy proceeds through the disproportionation reaction⁸



where K_s^+ represents a complexed or solvated potassium cation.

Metal solution samples were prepared under stringent high-vacuum conditions⁸ and ^{23}Na , ^1H and ^{13}C relaxation rates were measured using established procedures.

The frequency of the $^{23}\text{Na}^-$ resonance was found to be unchanged to within 0.5 p.p.m. on examining, over a range of temperatures, about 50 metal/12C4 solutions differing in both concentration and counter cation. All these Na^- resonance frequencies correspond to an increase in the electronic shielding

of the ^{23}Na nucleus relative to that in the gaseous sodium atom, Na_{gas}^0 , by ~ 2.5 p.p.m., after allowing for bulk-susceptibility corrections. To within the experimental errors arising from relating the solution measurements to the Na_{gas}^0 data, the shift in resonance frequencies is the same as that predicted (2.88 p.p.m.) from an accurate calculation⁷ of the shielding of a gaseous sodium anion, Na_{gas}^- , relative to that of Na_{gas}^0 . This indicates that both the 2p and 3s electrons of Na^- in solution interact very weakly, if at all, with their surroundings, the resonance frequency of the Na^- ion being diamagnetically shifted relative to that of Na_{gas}^0 by the additional shielding generated by the two, spin-paired 3s valence electrons.

The shielding of Na^- relative to Na_{gas}^0 should be contrasted with the substantial de-shielding of ~ 60 p.p.m. of Na_s^+ relative to Na_{gas}^0 , which is considerably larger than the 5.18 p.p.m. de-shielding⁷ of a gaseous sodium cation, Na_{gas}^+ , relative to Na_{gas}^0 . The additional de-shielding of Na^+ in the solution arises from the Ramsey paramagnetic contribution to the shielding generated by the interaction of Na^+ with the environment. Any such paramagnetic contribution is absent in the gaseous atoms and ions.

A detailed examination⁹ of the concentration and temperature dependence of the rates of ^{23}Na nuclear spin-lattice relaxation shows that the dominant, but still extremely inefficient, relaxation arises from the interaction of the nuclear electric quadrupole moment (eQ) of the ^{23}Na nucleus with fluctuating electric field gradients. The relaxation of nuclei with spin (I) $> 1/2$ is expected to be dominated by this quadrupolar mechanism¹⁰.

Nuclear spin-lattice relaxation rates (T_{1n}^{-1}) for Na^- in 12C4 are very much less than the value of $3,770 \text{ s}^{-1}$ for Na^+ in this solvent. The cation relaxation arises from the very large fluctuations in the electric field gradient caused by the asymmetric environment of the Na^+ ion resulting from its strong complexation by the solvent¹¹. The very small relaxation rate for Na^- in the solvent 12C4 is comparable with that reported in Table 1 for Na^+ in H_2O , which experiences an environment, the time average of which is centrosymmetric^{10,12,13}.

It is therefore of interest to compare the Na^- nuclear relaxation rate with those of other ions which are in a centrosymmetric environment^{10,12,13}. Note, however, that the experimental spin-lattice relaxation times of Table 1 are not, at this stage, directly

Table 1 Measured and scaled nuclear spin relaxation rates for ions in various solvents

Ion/solvent	(1)	(2)	(3)	(4)
	Measured T_{1n}^{-1} * (s^{-1})	Nuclear properties (I, eQ)	T_{1n}^{-1} scaled for: Electronic anti-shielding (β_2)	Viscosity (τ_{soln})
$\text{Na}^+/\text{H}_2\text{O}$	16.2	16.2	191	7,109
$\text{Na}^-/12\text{C4}$	3.33	3.33	3.33	3.33
$\text{Cl}^-/\text{H}_2\text{O}$	25	39	1.66	61.66
$\text{Cl}^-/\text{C}_2\text{H}_5\text{OH}$	1,300	2,047	86	716.7
$\text{Cl}^-/12\text{C4}$	14,100	22,260	935	935
$\text{I}^-/\text{H}_2\text{O}$	4,600	346	3.58	133
$\text{Cs}^+/\text{H}_2\text{O}$	0.075	817	15.5	577

* With the exception of relaxation rates in 12C4 these data are taken from refs 12, 13.

comparable because T_{1n}^{-1} is proportional, among other things, to the square of the nuclear electric quadrupole moment (eQ)², and depends on a theoretically well-established function of the nuclear spin I . Thus, column (2) of Table 1 reports the suitably scaled T_{1n}^{-1} values that would arise if all the ions had the same eQ and I as the ^{23}Na nucleus. This scaling (from column (1) to (2) in Table 1 uses theory of such firmly established validity¹⁰ that these numbers can be regarded as experimental results.

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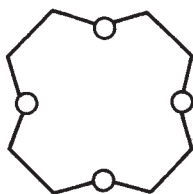


Fig. 1 1,4,7,10-tetraoxacyclododecane (12-crown-4).

The electric field gradient at the ^{23}Na nucleus generated by the environment is magnified by a factor $(1+\beta_2)$, the Sternheimer anti-shielding factor¹⁴, which originates from the additional electric field gradient generated by the electrons belonging to the ion. This additional electric field gradient arises because the external environment distorts the electronic charge distribution away from spherical symmetry. Since T_{1n}^{-1} is proportional to $(1+\beta_2)^2$, the results of column (2) of Table 1 can be scaled to yield the rates that would arise if all the ions had the same Sternheimer anti-shielding factor as Na^+ . The resulting data (column (3) of Table 1) are genuine reflections of the differences in strengths of the interactions of each nucleus with its environment external to the ion. Thus, these numbers now reflect the differences in the microscopic structure and microdynamics of the ions in solution.

In any theory of nuclear relaxation in liquids¹⁰, these microdynamics can be characterized by a correlation time, τ_c , defining the rate of fluctuations of the electric field gradient at the nucleus. For all of the ions except Cl^- in 12C4 and Na^+ , the molecules in the first solvation shell reorientate independently both of the ion and of each other^{12,13}. As this is the first report of the T_{1n}^{-1} data for Cl^- in 12C4, the details of the microdynamics responsible for this relaxation have not yet been elucidated.

For all of the Na^+ solutions, differing in concentration and counter cation, the activation energy ($22 \pm 1 \text{ kJ mol}^{-1}$) for the process responsible for the Na^+ spin-lattice relaxation was found to be essentially identical to that ($23 \pm 1 \text{ kJ mol}^{-1}$) for the process responsible for the ^{13}C and ^1H relaxation in the pure solvent. As the latter relaxation arises from reorientation of individual 12C4 molecules, our activation energies show that such independent solvent reorientation is the source of the fluctuations in the electric field gradient responsible for the Na^+ spin-lattice relaxation. Hence the correlation time governing the Na^+ relaxation is the same as that, τ_{solvs} , for the reorientation of individual uncomplexed solvent molecules in the neat liquid. This τ_{solvs} was derived from the values of T_{1n}^{-1} for nuclei such as ^{13}C and ^1H , whose relaxation is dominated by direct dipolar interactions between the spins. As the correlation time for neat 12C4 thus derived, 130 ps, is much longer than either that (15.6 ps) of $\text{C}_2\text{H}_5\text{OH}$ or that (3.5 ps) of H_2O , the scaled relaxation rates of column (3) in Table 1 for Na^+ relaxation in 12C4 would be expected to be much greater than those for the ions in the other two solvents if the strengths of the fluctuating electric field gradients were comparable. The much longer τ_{solvs} of 12C4 is consistent with the much greater viscosity⁹ (10 cP) compared with that of H_2O (1 cP).

The data in column (4) of Table 1 are derived by scaling those of column (3) by the factor τ_{solvs} (12C4), thereby generating the relaxation rates that the ions would have if they all had the same quadrupole moments, nuclear spins and Sternheimer factors as Na^+ , and were all in solvents of the same viscosity as 12C4. This scaling markedly increases the rates of column (3) in Table 1, except those for Na^+ and Cl^- in 12C4 yielding scaled relaxation rates which illustrate that the strength of the fluctuations of the environment experienced by the Na^+ ion is at least an order of magnitude smaller than those for all of the other systems.

Note that the 'raw' experimental relaxation rates of column (1) (which govern the NMR line width) might be incorrectly interpreted to suggest that the interaction of Na^+ with its environment in H_2O is only slightly greater than that of Na^+

with its environment in 12C4. However, the present scaled relaxation rates show that this is not the case, and they (column (4)) provide the first conclusive evidence that the sodium anion in these systems is considerably more decoupled from its environment than any of the other common ions. In particular, the interaction of Na^+ with its environment is ~ 280 times smaller than that of the Cl^- ion in the same solvent (12C4).

The chemical shift measurements show that Na^+ in 12C4 interacts so weakly with its environment that it behaves essentially as if it were in the gas phase. The nuclear relaxation data also show that Na^+ interacts only very weakly with its environment. We have shown not only that this interaction is much smaller than that of halide ions in a variety of solvents, but also that Na^+ interacts much more weakly with 12C4 than does the Cl^- ion. It would be inappropriate to attempt to explain here the reasons for this remarkable difference between Na^+ and halide ions.

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Distribution of atoms at the surface of liquid mercury

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Theoretical studies of the anisotropic distribution of atoms in the liquid/vapour interface have led to two conclusions. First, the density along the normal to the interface (the longitudinal one particle distribution function) has different forms in a liquid dielectric and a liquid metal. In a dielectric¹, the density decreases monotonically as one passes from the liquid to the vapour, while in a metal² the liquid/vapour interface is stratified for about three atomic diameters into the bulk liquid, Fig. 1 shows examples. Second, the transverse structure factor at the surface of a liquid (the Fourier transform of the in-plane distribution of pairs of atoms) is very similar to the bulk liquid structure factor except near the origin, the difference arising from long-range correlations in the surface¹. The very few experiments^{3,4} that can be interpreted in terms of the properties of the density along the normal to the liquid surface are in agreement with the first set of predictions. There have been no published reports on the transverse structure factor in the liquid/vapour interface. Here we describe the transverse structure factor of the room temperature liquid/vapour interface of mercury, determined using the method of grazing incidence X-ray diffraction⁵. Our experimental results are consistent with the above predictions.

The experiments were carried out at the Cornell High Energy Synchrotron Source (CHESS) using an apparatus that will be

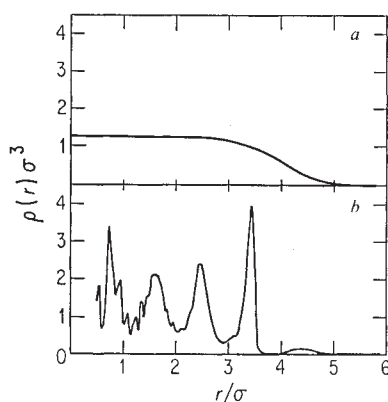


Fig. 1 The density (ρ) along the normal to the surface for: *a*, a typical dielectric liquid; and *b*, liquid Hg as determined in the Monte-Carlo simulations of D'Evelyn and Rice². The density and distance are put in reduced units through the atomic diameter σ . The coordinates chosen assume a free cluster configuration as actually used in ref. 2.

described in detail elsewhere. The geometry used is shown in Fig. 2. The sample is illuminated with X rays of wavelength λ at an angle ϕ which is much smaller than the critical angle for total external reflection, ϕ_c . Given that $\phi_c \propto \lambda$, in our experimental conditions the penetration depth of the X rays, $d \approx \lambda(2\pi)^{-1}(\phi_c^2 - \phi^2)^{-1/2}$, is independent of wavelength; for Hg it has the value 30 Å. The detector is set with vertical pick-off angle $\phi' \approx \phi_c$ and is scanned as a function of the azimuthal angle 2θ . Let $\mathbf{k}_0$ and $\mathbf{k}_d$ be the wavevectors of the incident and diffracted X rays. Since ϕ' is very small, the scattered wavevector magnitude is given by $|\mathbf{k}| \approx |\mathbf{k}_0 - \mathbf{k}_d| = 4\pi \sin \theta / \lambda$, the usual Bragg relation. This observation has the important consequence that, since the momentum transfer in the X-ray scattering is parallel to the surface of the liquid, only those atom-atom correlations in planes parallel to the surface are probed in our experiment.

We define the transverse structure factor (excluding polarization and inelastic X-ray scattering effects) by

$$S_{\parallel}(k) = \frac{\int w(\phi, \phi', z) S(k_{xy}, z) f^2(k) \rho(z) dz}{\int w(\phi, \phi', z) f^2(k) \rho(z) dz}$$

where $w(\phi, \phi', z)$ is a weight factor which describes the X-ray intensity at depth z , $S(k_{xy}, z)$ is the transverse liquid structure factor at z , $f(k)$ is the atomic structure factor and $\rho(z)$ scales as the average number of scatterers at depth z . The normalization is $S_{\parallel}(k) \rightarrow 1$ as $k \rightarrow \infty$.

Our sample was commercially obtained triply-distilled Hg. It was further purified by hot oxidation of impurities and their subsequent removal by filtering the liquid repeatedly through very long glass capillaries. The Hg was introduced into the beryllium-windowed stainless-steel ultra-high vacuum chamber through a capillary after the base pressure in the chamber had been reduced to 10^{-8} torr or less. Using the same conditions,

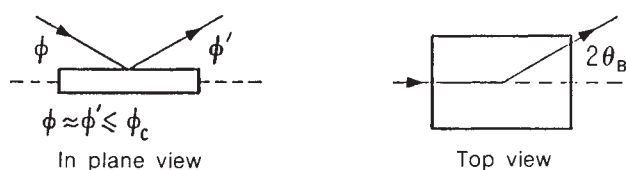


Fig. 2 Geometry of grazing incidence X-ray diffraction experiments.

previous experiments have shown that the liquid Hg surface remains clean for several days⁶. The 8- and 16-keV X rays used were reflected from a double-bounce channel-cut Si(220) crystal and deflected down onto the liquid sample by a Pt-coated float glass mirror, at an angle of incidence of 1.3 mrad. Horizontal slits were used to define the incident beam and the pick-off angle, while vertical Soller slits in front of the detector define the azimuthal angle 2θ to $\pm 0.15^\circ$. An intrinsic germanium energy-dispersive detector was used, which enabled us to separate X-ray fluorescence from X-ray scattering. Corrections for the instrument geometry, and for the decay of incident intensity as the storage ring current decays, were made by scaling the diffraction intensity to the intensity of the 10.1-keV Hg fluorescence line. In our experiment, the intensity of such a fluorescence line is independent of θ . The data were corrected for atomic, anomalous and Compton scattering and polarization (assuming the X-ray beam is plane polarized), and normalized using the Krogh-Moe method⁷.

Figure 3 shows the experimentally determined structure factor at 298 K and, for comparison, the structure factor for bulk liquid Hg at 293 K (ref. 8). The error in the bulk structure factor is predicted from counting statistics and is not greater than twice the line thickness in Fig. 3. Note the distinct features: $S_{\parallel}(k)$ for the interface is lower than $S_{\text{bulk}}(k)$ in the region from 1 to 2 Å^{-1} ; the trough between the first and second peaks in $S_{\parallel}(k)$ is slightly

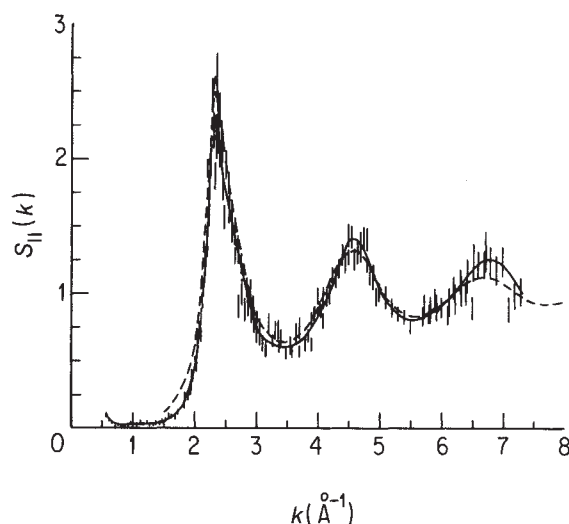


Fig. 3 The transverse structure factor for X-ray scattering from the surface of liquid Hg at 298 K. The vertical lines are out statistical error bars through which we have drawn a solid line to guide the eye. The dashed line is the bulk liquid structure factor at 293 K, from ref. 8.

deeper than in $S_{\text{bulk}}(k)$; the second peak amplitude in $S_{\parallel}(k)$ is slightly higher and narrower than the corresponding peak in $S_{\text{bulk}}(k)$; there is an apparent increase in $S_{\parallel}(k)$ as $k \rightarrow 0$ starting at $k \approx 1 \text{ Å}^{-1}$.

An examination of the temperature dependences of the structure factors of several bulk liquid metals⁷ shows that as the density increases, the low k onset of the first peak decreases and the peak amplitudes are increased. Our results imply that the surface of liquid Hg is more dense than that of the bulk. This further supports the predictions of a stratified liquid/vapour interface density profile; since the total elastic scattering intensity scales as the atomic density, $S_{\parallel}(k)$ will be dominated by the contributions from the high-density strata.

Kalos *et al.*¹ calculated the transverse structure function for the liquid/vapour interface of a model Lennard-Jones system

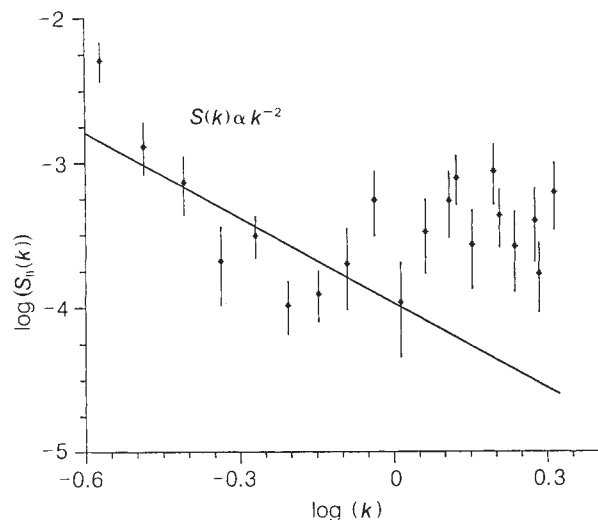


Fig. 4 A log-log plot of $S_{\parallel}(k)$ against k at low k .

with parameters of the potential chosen to describe Ar. They find a divergence in $S_{\parallel}(k)$ as $k \rightarrow 0$ with variation as k^{-2} . The theoretical interpretation of this divergence associates it with the existence of long-range correlations in the surface, the absence of any external force acting on the system and the non-vanishing of the derivative of density with respect to displacement along the normal. While our surface transverse factor is different from that of Kalos *et al.*¹ (their factor is given for a thin slice at $z=0$ and ours is integrated and averaged over a penetration depth), it is assumed that only the first or second uppermost layers will show any long-range transverse correlation; if present, the contribution of such a correlation to $S_{\parallel}(k)$ would overwhelm any contribution to $S_{\parallel}(k)$ from lower layers. Of course, our experiments are carried out in the Earth's gravitational field, which violates one of the conditions used in the theoretical analyses, but that field is so weak that, if everything else is the same, long (but not infinite)-range correlations should still be present.

Although the log-log plot in Fig. 4 shows that some scatter exists for the low k values of $S_{\parallel}(k)$, we believe the k^{-2} dependence of $S_{\parallel}(k)$ is a reasonable fit to our data. At least for the present, there is no reason to disbelieve the predicted k^{-2} dependence of $S_{\parallel}(k)$.

Our results are the first determination of the transverse-structure factor at the surface of a liquid. We expect that the grazing incidence X-ray diffraction technique and its neutron-scattering equivalent will be powerful tools for studying the variation of interface structure when a metal is alloyed, when a liquid supports a film or other structure and, in general, the interaction of a liquid interface and a contact medium.

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Efficient solar energy conversion with CuInS_2

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The high absorptivity associated with a direct energy gap in the optimum range for solar-energy conversion makes CuInS_2 a particularly promising material for efficient solar-energy conversion¹. Achieved solar-to-electrical conversion efficiencies have been limited to ~6% (refs 2–8). We report here a new heterogeneous polycrystalline $n\text{-CuInS}_2$ based semiconductor which has yielded conversion efficiencies of 9.7% in an electrochemical cell. The high photoactivity is also evident in a Schottky barrier solar cell configuration. The origin of the improved efficiency is attributed to impurity scavenging by In spheres resulting from a modified vapour/liquid/solid (VLS) growth process^{9–11} and the influence of the acidic iodine iodide electrolyte on the cell performances.

Samples with increased In excess exceeding the single-phase region in the phase diagram¹² were prepared. CuInS_2 was synthesized from the elements (6 N/Ventron) in a mole ratio of 0.95, 1.05 and 2, respectively. In the growth process, the molten Cu–In alloy reacted with S at $T=1,175^\circ\text{C}$ to yield the new material. The resulting boule consists of large-grain polycrystalline material and exhibits a comparably porous structure in its interior. The samples with the highest photoactivity are found in this region inside the boule. Sizes range between 1 and 5 mm². A typical surface is shown in Fig. 1. The morphology is characterized by extended steps typical for VLS growth¹³. In addition, small In spheres and In_2S_3 and Cu_{2-x}S occlusions are found and extend up to the surface on some samples.

For photoactivity measurements, electrodes were prepared as described earlier¹⁴ using In–Ga alloy as the ohmic back contact. Figure 2a shows the current/voltage characteristic under illumination with simulated AM1 direct radiation (85 mW cm^{-2}) (Oriel Solar Simulator) of $n\text{-CuInS}_2$ in contact with acidic iodine-iodide electrolyte. Because of the comparably small electrode, area determination was done by scanning laser spot analysis^{15–17} to measure the photoactive area accurately. In a two-electrode experiment, the cell converts light to electricity

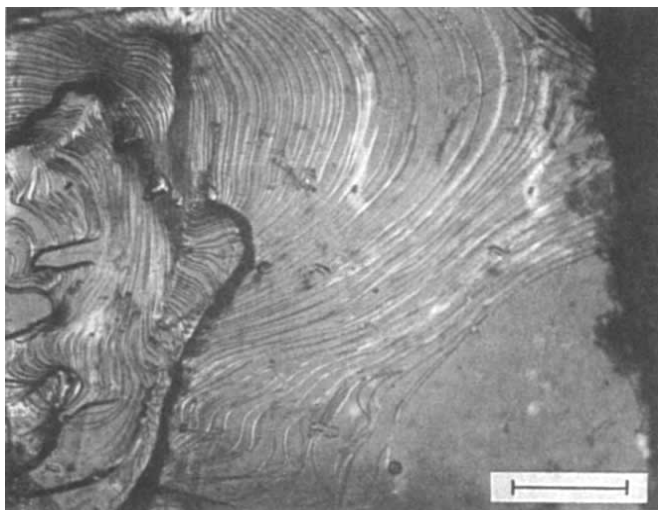


Fig. 1 Electron micrograph of a typical melt grown CuInS_2 sample with In excess; note terraces indicative of VLS growth and In spheres. Scale bar 200 μm .

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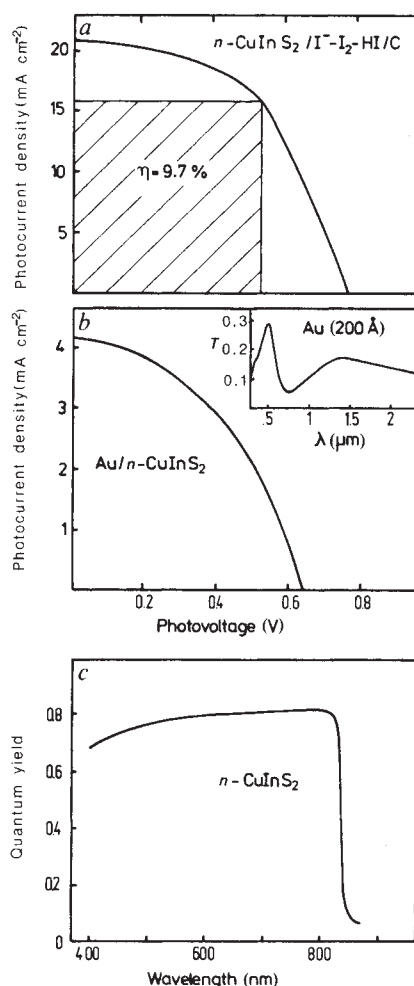


Fig. 2 Photoactivity of heterogeneous CuInS_2 . *a*, Output power characteristic of the $n\text{-CuInS}_2/\text{HI-I}^-_2\text{-C}$ solar cell; electrolyte: 2 M HI, 2.5 M CaI_2 , 50 mM I_2 ; illumination: Oriel Solar Simulator, AM 1 direct (85 mW cm^{-2}). *b*, Output power characteristic of the $\text{Au}/n\text{-CuInS}_2$ solar cell; evaporation of front metal contact after potential cycling in $\text{HI-I}^-_2\text{-I}_2$ redox electrolyte; illumination: 64 mW cm^{-2} natural sunlight. *c*, Spectral dependence of the quantum yield; electrolyte: 2 M HI, 2.5 M CaI_2 , 50 mM I_2 ; potential: 0.1 V (SCE).

with an efficiency of 9.7%. The short-circuit photocurrent is stable for $\sim 20,000 \text{ Cb cm}^{-2}$. During the long time testing period no decrease in photovoltage has been found. Figure 2*b* shows the photocurrent/voltage characteristic of a $\text{Au}/n\text{-CuInS}_2$ junction obtained in natural sunlight of 64 mW cm^{-2} (Kipp-Zonen global detector). Before metal evaporation, the sample was polarized in $\text{HI-I}^-_2\text{-I}_2$ electrolyte ($-0.1 \text{ V} \rightarrow -0.6 \text{ V}$ (SCE)) which resulted in an improved cell output compared with untreated electrodes. The spectral dependence of the transmission of the 200 Å thick Au film is displayed in the inset of Fig. 2*b*. The light attenuation for photon energies above the CuInS_2 band gap ($\sim 830 \text{ nm}$) can be estimated by the area under the transmittivity curve. Since the total transmission is 25% in this energy range, a correction factor of ~ 4 for efficiency calculation is obtained. This would result in a solar-to-electrical conversion efficiency of $\sim 7.5\%$.

The spectral dependence of a typical quantum yield is shown in Fig. 2*c*. The experiments were performed in the standard potentiostatic arrangement with a carbon counter electrode and a calomel reference electrode. The quantum yield was obtained by calibrating a photocurrent spectrum with the photon flux of a He-Ne laser at 633 nm. Despite the absorptivity of the iodine-iodide solution and the fact that the distance between electrode and cell window was not optimal, the values exceed 0.8.

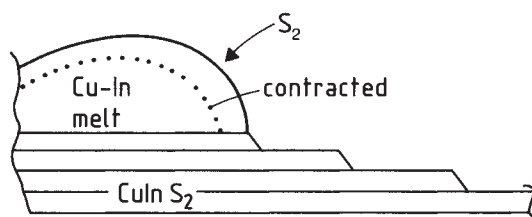


Fig. 3 Schematic representation of VLS growth of highly photoactive CuInS_2 (see text).

The photoactivity improvement of the new material can be interpreted based on details of the growth process. An additional advantageous influence of the selected electrolyte is also evident, particularly from the preparation experiments before metal evaporation indicating that surface optimization contributes to the high cell output¹⁸⁻²⁰. In VLS (vapour/liquid/solid), growth of CuInS_2 , a Cu-In melt exists at a given temperature which is supersaturated by gaseous S_2 leading to abrupt contraction of the melt and resulting in the growth of a new terrace^{21,22}. This process, schematically shown in Fig. 3, explains the surface morphology shown in Fig. 1. When the growth is interrupted at a time when not all of the remaining sulphur has reacted, the In excess leads to the observed residual In spheres found on these samples (Fig. 1).

The spheres seem to act as a reservoir for impurities, as spark source mass spectrometry (SSMS)²³ and inductively coupled plasma (ICP)²⁴ analyses show a ~ 40 times increased Fe content of the metal spheres compared with the neighbouring material¹¹. Fe probably substitutes In in the host compound leading to the localized formation of CuFeS_2 which acts as a deep trap. Time resolved microwave conductivity (TRMC)²⁵ and photoluminescence (PL) experiments reveal a drastic reduction in the trap density in the energy gap compared with differently prepared CuInS_2 (ref. 10). The prevailing effect of the material heterogeneity thus seems to be the creation of a reservoir for the most deleterious impurities with a material (In) with favourable segregation coefficients, similar to the process of zone refining²⁶.

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Vertical profile of lead isotopic compositions in the north-east Pacific

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Ratios of lead isotopes (^{204}Pb , ^{206}Pb , ^{207}Pb and ^{208}Pb) may be used to distinguish leads from different sources in sea water¹⁻³ in a manner similar to neodymium isotopes⁴⁻⁸. The following data from the north-east Pacific include the first vertical profile measurements of lead isotopic compositions in sea water. These preliminary data indicate that lead in offshore (48°N , 141°W) north-east Pacific surface waters ($^{206}\text{Pb}/^{207}\text{Pb} = 1.164$) was derived from aeolian inputs of Asian (Japanese) industrial leads ($^{206}\text{Pb}/^{207}\text{Pb} = 1.160$) (M. Murozumi, personal communication), but that lead in water immediately (400 m) below it ($^{206}\text{Pb}/^{207}\text{Pb} = 1.211$) was derived from North America industrial leads ($^{206}\text{Pb}/^{207}\text{Pb} = 1.222$)⁹. This remarkable contrast in isotopic compositions over such a relatively short distance reveals the potential of lead isotopic compositions to resolve the importance of horizontal transport processes on the lead cycle in the oceans, which were recently proposed to partially account for its oceanographic variability in the North Pacific¹⁰.

Seawater collections were obtained along a transect from 48°N , 125°W to 48°N , 141°W in August 1980 (Fig. 1). Surface water samples were obtained from a workboat deployed away from the research vessel, *CSS Parizeau*. Subsurface samples were collected with Caltech 10-1, deep-water samplers¹¹. Those samples were then transferred to bottles for storage at 4°C within a shipboard clean laboratory¹², using trace-metal clean procedures¹¹.

Isotopic composition measurements by thermal ionization mass spectrometry were made with samples (41) after extraction with the dithizone-chloroform procedure described elsewhere³, as were the lead concentration measurements^{11,13}. Coefficients of variation of individual ratios typically were: $^{208}\text{Pb}/^{206}\text{Pb} \leq 0.2\%$; $^{207}\text{Pb}/^{206}\text{Pb} \leq 0.01\%$; and $^{206}\text{Pb}/^{204}\text{Pb} \leq 0.3\%$. Contaminant lead corrections calculated from summations of individually determined contributions of contamination by reagents and containers were $\leq 2\%$ of the sample lead.

The different sources of lead in north-east Pacific surface waters are established by the differences in their isotopic compositions listed in Table 1, especially the $^{206}\text{Pb}/^{207}\text{Pb}$ ratios which are most commonly used as indicators of different lead sources in sea water^{2,3}. Isotopic compositions of lead aerosols from industrial sources impacting those waters are also listed in Table 1 for comparison. The $^{206}\text{Pb}/^{207}\text{Pb}$ ratios in these north-east Pacific surface waters range from 1.164 in the offshore (48°N , 141°W) waters (Station P-12) to 1.204 in the coastal (48°N , 127°W) waters of the Juan de Fuca Straits (Station P-17). This range in $^{206}\text{Pb}/^{207}\text{Pb}$ ratios over 1,900 km in the north-east Pacific is greater than that (1.174–1.196) over 3,300 km in the central (19°N , 158°W to 15°S , 150°W) Pacific³. It substantiates reports that the lead in offshore surface waters of the north-east Pacific is primarily derived from Asian industrial lead ($^{206}\text{Pb}/^{207}\text{Pb} = 1.160$), and the lead in surface waters of the Juan de Fuca Straits is primarily derived from North American industrial lead ($^{206}\text{Pb}/^{207}\text{Pb} = 1.222$)^{2,3,11,13}. It is also consistent with ^{210}Pb data from the Pacific, which indicate the predominance of aeolian inputs in oceanic surface waters and of both aeolian and fluvial inputs in coastal waters¹⁴⁻²⁰.

The vertical gradient in lead isotopic compositions in the

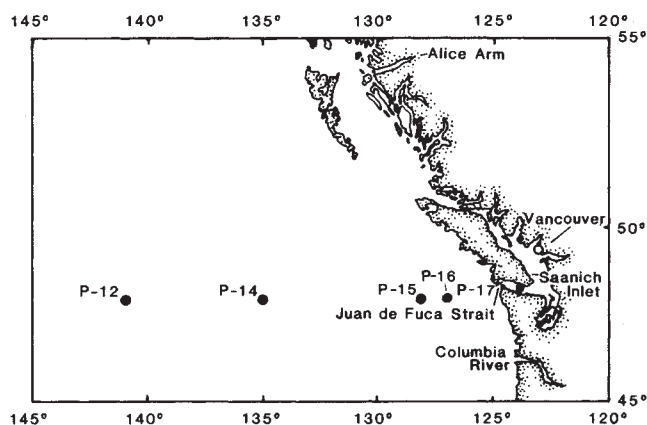


Fig. 1 Sampling locations in the north-east Pacific.

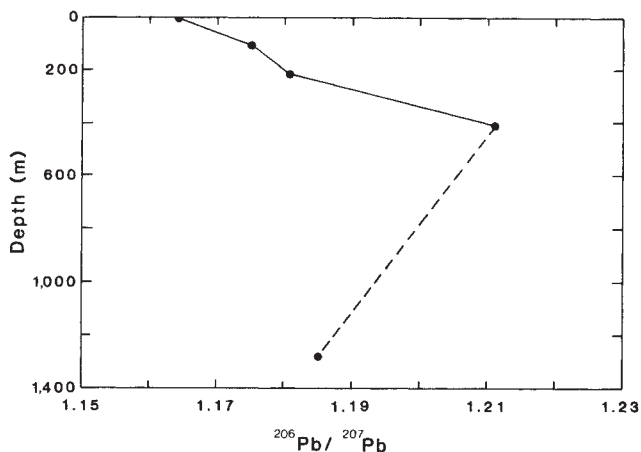


Fig. 2 Lead isotopic ratios ($^{206}\text{Pb}/^{207}\text{Pb}$) at 48°N , 141°W in the north-east Pacific.

offshore (48°N , 141°W) waters (Station P-12) is even more pronounced. There the $^{206}\text{Pb}/^{207}\text{Pb}$ ratio increases from 1.164 in the surface waters to 1.211 at 400 m, and then decreases to an intermediate value at greater depth (Fig. 2). The relatively large magnitude of this range over the upper 400 m is illustrated by comparison with the previously cited ranges of $^{206}\text{Pb}/^{207}\text{Pb}$ ratios in surface waters over 1,900 km in the north-east Pacific and over 3,300 km in the central Pacific. For example, the $^{206}\text{Pb}/^{207}\text{Pb}$ vertical gradient ($\Delta^{206}\text{Pb}/^{207}\text{Pb} : \Delta\text{km}$) is more than 19,000 times greater than the horizontal gradient in the central Pacific.

This pronounced vertical gradient in lead isotopic compositions indicates the potential of lead isotope systematics to delineate the element's biogeochemical cycle in the ocean. Specifically, these data indicate that lead isotopic compositions can establish the significance of horizontal transport processes within the ocean, which have been proposed to account for the parallel vertical concentration gradients of lead, bismuth and plutonium in the North Pacific¹⁰. For example, the subsurface (728 m) lead concentration maximum (68 pmol kg^{-1}) in these subarctic waters is consistent in size and shape with those in more temperate waters of the north-east Pacific^{11,13,21,22}, but the apparent North American isotopic composition of that lead precludes it from having the western Pacific origins which have been proposed to account for the lead concentration maxima in the more southern waters¹⁰.

Unfortunately, there were neither samples for additional lead measurements nor sufficient oceanographic data obtained on the cruise to resolve adequately the lead inputs at this oceanographically-complex location. Consequently, the subsurface lead may reflect a horizontal input of North American lead

Table 1 Isotopic compositions of lead in the north-east Pacific and in aeolian inputs from industrial sources

	Lat.	Long.	Depth (m)	Isotopic composition			Concentration (pmol kg ⁻¹)
				206/207 ($\bar{x} \pm 1s$)	206/208 ($\bar{x} \pm 1s$)	206/204 ($\bar{x} \pm 1s$)	
Seawater samples							
Station							
P-12	141°00' W	48°00' N	0	1.164±0.0023	0.4709±0.0022	17.39±0.70	27.0/25.5*
			106	1.175±0.0019	0.4785±0.0010	18.36±0.05	45.4
			216	1.181±0.0005	0.4824±0.0003	—	59.8
			404	1.211±0.0017	0.4965±0.0023	18.98±0.19	62.7
			728	—	—	—	68.0
			1,247	—	—	—	27.0
			1,282	1.185±0.0046	0.4822±0.0025	19.05±0.53	20.7
			1,650	—	—	—	18.8
			1,980	—	—	—	14.4/15.4
			2,714	—	—	—	11.1
			3,249	—	—	—	12.5
			3,945	—	—	—	10.1
P-14	135°00' W	48°00' N	200	1.191±0.0048	0.4884±0.0029	18.35±0.09	60.3/60.3*
P-15	128°00' W	48°00' N	200	1.180±0.0006	0.4826±0.0005	—	91.7
			400	—	—	—	125.0
P-16	127°00' W	48°00' N	0	1.204±0.0005	0.4915±0.0032	—	7.7/13.9*
P-17	124°00.8' W	48°21.8' N	0	—	—	—	31.4
Aeolian inputs							
Western USA ⁹				1.22	0.496	19.3	—
Asia/Japan USA [†]				1.16	0.479	17.8	—
Western Canada ²				1.16	0.480	18.4	—

* Concentrated acid digestion as detailed elsewhere¹⁴.

† M. Murozumi, personal communication.

from a southern incursion of the California Undercurrent, the diagenic mobilization of shelf sediments, the nepheloid transport of unconsolidated shelf sediments, or the subsurface decomposition of plankton in coastal waters. Additionally, that lead may reflect a vertical input from a previous aeolian flux of North American lead to the surface waters. Although those potential inputs cannot be resolved with these preliminary data, their multiplicity illustrates the need for additional isotopic data to distinguish different sources of lead in the ocean.

Therefore, the principal significance of these data is that they demonstrate the potential of stable lead isotopic compositions as geochemical tracers in the ocean. The pronounced ²⁰⁶Pb/²⁰⁷Pb gradient in this preliminary vertical profile indicates that those ratios can be utilized to determine the significance of different inputs and transport processes on the oceanic lead cycle. It also indicates that those ratios may be utilized as tracers of water masses, as well as of biogeochemical transformations of lead and its analogues (such as calcium) within those water masses.

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Basal metabolic rate and energetics of reproduction in therian mammals

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Traditionally, the fact that reproduction in eutherian (=placental) mammals tends towards prolonged intrauterine development and short lactation has been interpreted as an evolutionary advance over the metatherian (=marsupial) short gestation and prolonged lactation¹. However, it has recently been postulated that marsupial reproduction involves low initial energy investment and may be advantageous by minimizing energy loss if conditions necessitate early termination^{2–5}. Moreover, because marsupials have basal metabolic rates (BMRs) 30% lower than those of most eutherians^{6,7}, it has been suggested that daily and total energy expenditures during reproduction may also be lower⁸. We have now tested the predictions that low BMR is maintained during reproduction and that initial investment is lower in marsupials. Using indirect calorimetry, we have made the first longitudinal measurements of energy expenditure during reproduction for a marsupial and for two eutherian species with low BMRs⁹. We find that initial investment is lowest in a eutherian and total energetic expenditures were greatest for the marsupial. We also find that, relative to BMR, all three species have increased mean maternal resting metabolic rates (RMRs) during both gestation and lactation; this is the first evidence that mammals with low BMRs can substantially elevate metabolism for prolonged periods during gestation and lactation.

We measured oxygen consumption ($\dot{V}O_2$) and body temperature (T_b) in five South American short-tailed opossums (*Monodelphis domestica*, Metatheria: Marsupialia: Didelphidae), three elephant shrews (*Elephantulus rufescens*,

Table 1 Comparison of incremental and absolute costs of reproduction among wild mammals

Species	Mass (g)	Litter size	BMR (% expected)	kcal per day	Gestation		Lactation		Total		Increment		% Increase in mother's % Kleiber		Ref.
					Duration	X-cost	Duration	X-cost	kcal total	Use per young	kcal total	Use per young	Gest.	Lact.	
Order Marsupialia															
<i>Monodelphis domestica</i>	82.0	8	64	6.8495	14	126	8.7	56	316	21.6	1,333	167	853	107	25
<i>Didelphis virginiana</i>	2,240.0	7	76	97.4941	14	—	—	85	—	—	—	—	—	15.0	
Order Macroscelidea															
<i>Elephantulus rufescens</i>	84.1	1	82	8.9720	57	122	10.9	15	184	16.5	872	872	226	226	
Order Insectivora															
<i>Echinops telfairi</i>	140.6	5	28	4.5043	60	183	8.2	23	539	24.3	1,054	211	681	136	
<i>Suncus murinus</i>	30.2	3	139	7.0550	30	—	—	18	248	17.5	—	—	—	—	26
Order Rodentia															
<i>Sigmodon hispidus</i>	125.8	5	95	14.0593	26	154	21.6	12	296	41.7	1,084	217	560	112	
<i>Microtus arvalis</i>	25.0	4	345	15.1968	18	118	17.9	16	255	38.7	941	235	419	105	
<i>Neotoma floridana</i>	237.0	3	146	34.7450	35	—	—	22	—	—	—	—	—	—	
<i>Reithrodontomys megalotis</i>	7.7	3	127	2.3129	—	—	—	21	—	—	—	—	—	—	*

BMR = per cent of expected as calculated from Kleiber⁹. kcal per day = [resting oxygen consumption (ml h^{-1}) $\times$ 0.0048 kcal ml^{-1} oxygen $\times$ 24 h] + kcal stored as mass in young. X-cost = incremental cost over BMR, as a multiple of BMR, per day. Total incremental cost = duration $\times$ X-cost. Per cent increase in mother's % Kleiber = (mean % Kleiber)/(BMR % Kleiber).

* S.D.T., unpublished data.

Eutheria: Macroscelidea: Macroscelididae) and one tenrec (*Echinops telfairi*, Eutheria: Insectivora: Tenrecidae). Earliest marsupials are thought to have been reproductively similar to small modern didelphids¹⁰ and tenrecs are among the most primitive of extant eutherians¹¹. To this extent the comparison reflects the potential for energetic differences having been involved in the evolutionary divergence of the two groups. Respirometry allowed maternal metabolism to be separated from that of the offspring during lactation, avoided the inclusion of activity costs usually included in studies of caloric intake, and thus allowed us to distinguish among net investment (growth of young), the cost of allocating that investment (mother's respiration) and maintenance costs for the growing litter (litter's respiration). Specific basal (or resting) metabolism ($\dot{V}\text{O}_2 \text{ g}^{-1} \text{ h}^{-1}$) was determined within thermoneutrality for each female before reproduction (BMR), during gestation (RMR_g), during lactation (RMR_l) and after weaning (BMR).

During gestation, females were placed in a Perspex chamber and submerged in a circulating water bath for at least 2 h at a maximum frequency of once weekly; room air was metered through the chamber at 40–150 ml min^{-1} . During lactation, measurements were made first on the mother and young together, then the young were removed and the female was immediately measured alone. $\dot{V}\text{O}_2$ was determined using an open-circuit respirometry system with a Beckman G-2 oxygen analyser calibrated using nitrogen gas¹². Animals were 6–12 h post-absorptive during all measurements and all measurements were made during the inactive phase of the day. We assumed a respiratory quotient of 0.8 in calculating $\dot{V}\text{O}_2$ (ref. 12). Because specific metabolism varies with body mass, we expressed the specific basal or resting metabolic rate as a proportion of that predicted by Kleiber⁹ for a non-reproductive eutherian of the same body mass. This was intended only to simplify comparisons between individuals and species of different mass. Oxygen consumption was converted to kcal per day assuming 0.0048 kcal per $\text{cm}^3 \text{ O}_2$ consumed. The energy content of the young was assumed to be 0.87 kcal per g wet body mass¹³.

RMR increased throughout reproduction in all three species (Fig. 1). RMR_l of female opossums plus offspring peaked immediately before weaning (50–56 days; M. Roberts, unpublished data) at 189% of Kleiber's predicted rate, when mean maternal T_b increased from 35 to $>36^\circ\text{C}$. Between 35 days post-partum and weaning, female RMR_l of *Monodelphis* was within 15% of the predicted value, peaking during the last week of lactation, at 106% of Kleiber, before declining to near pre-reproductive BMR. Mean RMR_l of female *E. rufescens* was

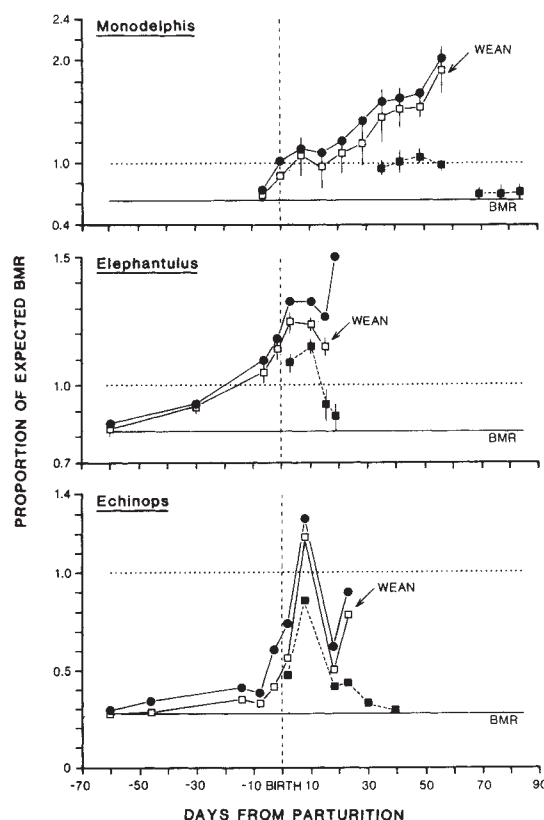


Fig. 1 Mass-independent rates of energy expenditure, expressed as a proportion of that expected from total body mass⁹ of mother alone (solid squares), mother with young (open squares), and mother with young with rate of energy storage as growth of litter added to oxygen consumption (solid circles); symbols before birth reflect conception to birth, those after birth reflect birth to weaning. Dotted horizontal line identifies Kleiber's⁹ predicted eutherian BMR. Solid horizontal line identifies mean BMR of non-reproductive females. Vertical bars denote ± 1 s.d. of the mean. Values calculated from $\dot{V}\text{O}_2 \text{ h}^{-1}$ times 0.0048 kcal per ml O_2 and assuming 0.87 kcal per g wet mass of growing offspring¹³. Values are per 24-h day.

Table 2 Proportion of incremental energy and total incremental energy expended during reproduction as a function of relative and absolute time from conception

	Proportion of CW time					
	0.25	0.50	0.75	0.85	0.90	0.95
<i>Elephantulus</i>						
Days from conception	18	35	53	60	63	67
Proportion of energy	0.029	0.141	0.435	0.638	0.743	0.889
Incremental kcal	7	31	97	145	168	201
Cumulative effort	0.02	0.09	0.202	0.262	0.29	0.327
<i>Echinops</i>						
Days from conception	21	42	62	71	75	79
Proportion of energy	0.044	0.155	0.397	0.712	0.822	0.910
Incremental kcal	30	106	270	484	560	619
Cumulative effort	0.322	0.558	0.953	1.494	1.635	1.719
<i>Monodelphis</i>						
Days from conception	17	35	52	59	62	66
Proportion of energy	0.046	0.166	0.445	0.626	0.730	0.852
Incremental kcal	38	142	381	536	625	729
Cumulative effort	0.339	0.608	1.07	1.321	1.456	1.604

CW, conception to weaning time. Incremental kcal = (RMR kcal per day of mother and young plus kcal stored as growth) - (BMR kcal per day of non-reproductive female). Cumulative effort = (incremental kcal per day/BMR of non-reproductive female) days from conception.

higher than predicted while that of *E. telfairi* was lower than predicted. These increases in specific metabolism were not due to separation anxiety; measurements made on the litters alone added to those for the mother alone were $\pm 6.5\%$ of values for mother and young measured together. Female plus offspring of both eutherians showed a similar increase in RMR over non-reproductive BMR, but peak RMR₁ was lower than that of the opossum: 125% of Kleiber's predicted in *E. rufescens* and 118% in *E. telfairi*. Lowest tenrec RMR₁ corresponded with maternal hypothermia, paralleling thermoregulatory patterns in resting non-reproductive tenrecs (S.D.T. and M.E.N., unpublished data) and involving a 3–5 °C reduction in T_b from 35–36 °C.

The proportion of total energy expended from conception to weaning, including that stored as growth of the young, which was allocated to pregnancy, reflected relative length of gestation and degree of neonatal development: 0.71 in the precocial *E. rufescens*, compared with 0.47 in *E. telfairi*, and 0.09 in *M. domestica*. Mean RMR₂ of *M. domestica* was not significantly greater than the non-reproductive BMR in the same individuals ($U = 40$, d.f. = 16, $P > 0.05$), although $\dot{V}O_2$ rose as female mass increased (~5 g) during pregnancy (Fig. 1).

Comparison of *E. rufescens* and *M. domestica*, with their similar adult body mass and conception to weaning time, revealed that total energy expenditure (kcal) and incremental energy expenditures (exceeding that required for BMR of a non-reproductive female of the same mass) during reproduction were greater in the marsupial. Higher incremental costs in *M. domestica* were related to greater reproductive effort and a comparatively high RMR₁ of the female plus offspring during late lactation. Although almost twice as large in size, total energy expenditure during reproduction by *E. telfairi* was lower than in *Monodelphis* (Table 1), reflecting maternal torpor and a slow increase in RMR from an extremely low BMR. Similarly, when *E. telfairi* is compared with a precocial rodent, *Sigmodon hispidus*, which has the same litter size and similar body mass¹³, the difference in total energy expenditure during reproduction is surprisingly small given the divergence in duration of maternal care and BMR (Table 1). The similarity stems in part from the compensatory increase in tenrec maternal RMR towards Kleiber's predicted value, which the BMR of *S. hispidus* approximates with no increase in maternal RMR during reproduction¹³.

Initial energy investment in reproduction, as defined by the incremental energy expended during the first 25% of parental care, was lower in the two eutherians than in *M. domestica*.

Through the first 75% of the duration of parental care, all three species expended about 40–45% of their total energy allocation to reproduction and all deferred over 29% of their energy expenditure until the last 15% of maternal care (Table 2). Despite great differences in energetic effort, all species showed a gradual increase in energy expenditure regardless of allocation of time or energy to either gestation or lactation (Table 2).

Our data reveal no advantages to either marsupial or eutherian reproduction with respect to total, daily or temporal patterning of energy expenditure. Comparison of longitudinal energy expenditure during reproduction suggests a continuum based not on phylogeny but on relative BMR. Thus, our three low BMR species, of diverse phylogeny, show a large increase in mean maternal RMR during reproduction whereas for species with a BMR near that predicted, such as wild eutherians (Table 1), laboratory mice¹⁴ and rats^{15,16}, mean maternal RMR is virtually the same as BMR. Our calculations from limited data for another marsupial, the North American opossum (*Didelphis virginiana*; Table 1), also show RMR₁ values greater than BMR. Because species with high BMRs show little increase in reproductive RMR, we suggest that high RMRs are either advantageous or necessary for mammalian reproduction and that at least some mammals with low BMRs can compensate during reproduction. The increased mean maternal RMRs of our study are distinct from those reported for livestock^{15–17}, chiefly because most livestock have not been measured when post-absorptive; thus, these elevated maternal RMRs reflect the heat of feeding¹⁷ associated with increased ingestion to support larger body mass during reproduction. Data from post-absorptive livestock agree with those in Table 1: fasted, lactating cows (BMR 87–120% predicted) have RMR₁s equal to BMR and fasted, lactating goats (with low BMRs, 70–75% predicted) have RMR₁s 12–15% above BMR¹⁶. Humans have BMRs near the predicted value and, whether fasted or fed, show little or no difference between average reproductive and non-reproductive rates of metabolism^{15,16,18}. Interspecific variation in both BMR and the ability to elevate RMR during reproduction could be responsible for the wide variation in pre- and postnatal growth rates, and the interaction of reproductive RMR with duration of gestation and lactation could be associated with 'intermediates' along the continuum from altricial to precocial neonates^{19,20}. If the latter proves correct, developmental stage (or relative mass) at birth will be more accurately predicted by the interaction of rate of metabolism and duration of gestation. Our study also supports recent suggestions that the predicted BMR reflects an advantageous or essential energetic regime for therian reproduction^{21–24}, from which deviations represent adaptation to small body size²⁴ or food habits²¹.

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Enhanced detection in the aperture of focal attention during simple discrimination tasks

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There is increasing evidence that it is possible to shift an aperture of focal attention to a position in visual space independent of fixation and that this can be done much faster than the eyes are able to move¹⁻⁴. Recently, we showed⁴ that such serial scrutiny by the aperture of focal attention is required before an observer is able to tell what a target is (for example, to know whether the orientation of a line segment is horizontal or vertical). Here we considered whether attention directed towards a specific position in the visual field for an orientation discrimination task improves performance on a simple detection task in the area to which attention is directed. We found that a small test flash could be detected when it was positioned near a peripheral line target presented briefly, if the orientation of the target had to be identified. The test flash could not be detected when presented at some distance from the same target or when another target had to be identified. This enhancement implies that even simple identification tasks such as orientation discrimination are not performed passively by the visual system.

Against a dark field, we presented two bright targets each extending 0.7 degrees of arc in length (horizontal or vertical line segments; or the letter L or T), one in the position where the observer was fixating, the other at 4° eccentricity from the fixation marker, but in any random direction from this marker. Observers were asked to identify only one of the targets (either peripheral or central) in a given block of trials, and were told that the peripheral target would always be at the same eccentricity but in any random direction. Presentation time was short (10 ms) to avoid a second fixation on the target by eye movement, and a mask was applied at a specific time, termed stimulus onset asynchrony (SOA), after target onset, in order to limit processing time (see Fig. 1a). Simultaneously with this target identification task, we also presented a small test flash with a 4 arc min diameter lasting 10 ms, at various positions across the visual field, and asked observers to report the presence or absence of the test flash, by pressing one of four keys: two for presence or absence of the test flash when target A was identified and another when target B was identified. Detection errors were signalled by ringing of a bell on the computer that ran the experiments. The test flash and the targets were masked at different SOAs so that performance on the two tasks could be controlled independently. The test flash appeared on 66% of the trials, and was presented at positions along two cardinal directions from the peripheral target: on the perimeter of an imaginary circle of the

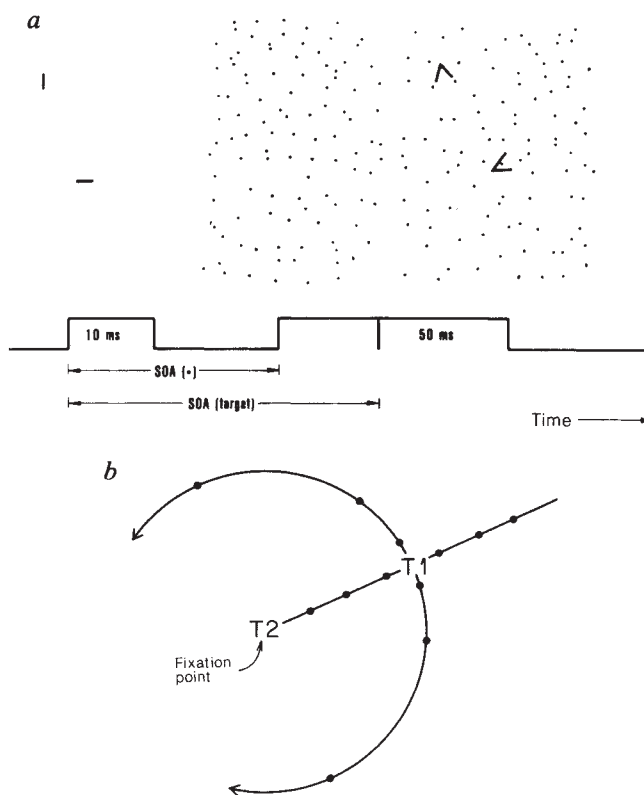


Fig. 1 *a*, The stimulus used in our experiments. The observers were presented first with a pair of targets, one at the fixation point (here a horizontal line) and another at 4° eccentricity. A small dot, the test flash, shown here near the vertical peripheral target, was presented on 66% of the trials. This pattern was presented for 10 ms then masked by random dots after a certain time, SOA(-), and after a longer time, SOA(target), a pair of randomly oriented 'V' symbols were presented at the locations of the targets for 50 ms. A fixation cross was presented at the position of the central target before each trial began, but was removed 50-100 ms (randomized between trials) before the stimulus onset. *b*, Possible locations of the test flash relative to the discrimination target T1. Fixation point is on T2, where the central target was presented. T1 represents the peripheral target; solid lines represent the two cardinal directions from T1 that were used for presenting the test flash. The test flash could be placed in one of 12 positions (indicated by the solid circles). These positions are relative to T1; when T1 appeared in another direction all the possible positions would rotate in this direction. We used the same configuration for experiments involving central and peripheral target discrimination.

same eccentricity as the peripheral target or on a line running from the central target to the peripheral target and beyond (see Fig. 1b). In each block of 50 trials the test flash could appear in any of the four directions and at any of three distances from the discrimination target. As observers had to identify only one of the targets in a given block of trials, we could compare detection rates for the test flash when the peripheral or the central target was identified, thus isolating changes in detection rate caused by the identification processes.

The results of experiments in which observers had to identify the peripheral target are shown in Fig. 2a. The results are presented as the difference between the frequency of correct detection when the test flash was presented, $P(\text{detect})$, and the false alarm rate, $P(\text{FA})$, representing the frequency of observers falsely reporting the presence of the test flash. As the distances between the test flash and the discrimination target were varied in each block of trials, the same false alarm rate was measured for all distances, so an increase in $[P(\text{detect}) - P(\text{FA})]$ reflects

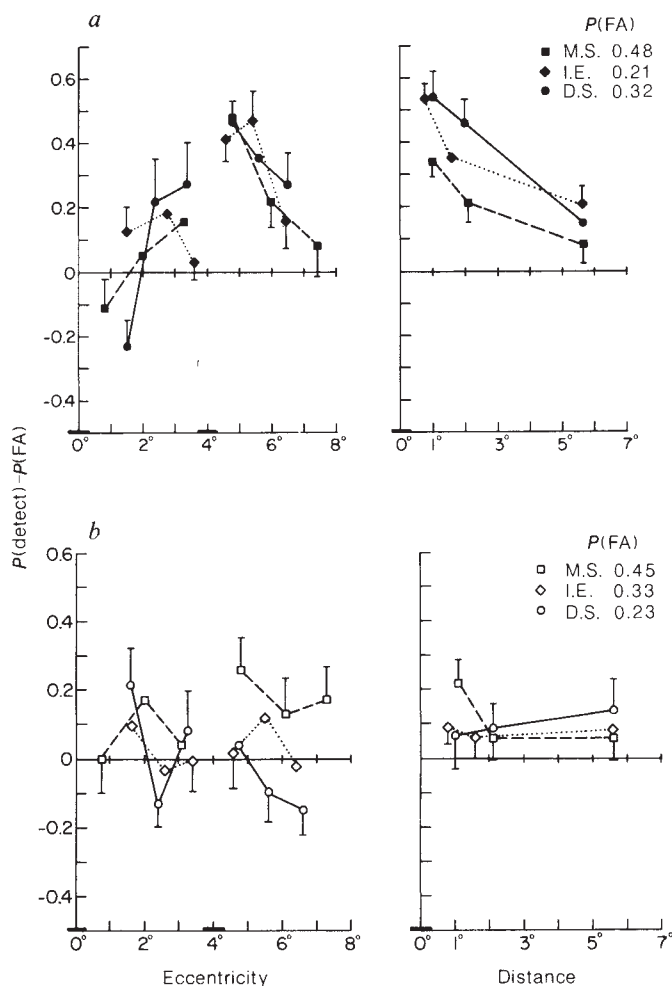


Fig. 2 *a*, Data from experiments in which observers had to identify the peripheral target. Plot on the left shows the dependence of $P(\text{detect}) - P(\text{FA})$ on the radial distance of the test flash from the fixation point, the peripheral target here being at 4° eccentricity. Plot on the right shows the dependence of $P(\text{detect}) - P(\text{FA})$ on the distance from the peripheral target along an equi-eccentricity circle; data for the clockwise and anticlockwise directions were combined. Data shown are for three observers: M.S. and D.S., who performed an orientation discrimination task with $\text{SOA}(\text{target}) = 60$ ms for M.S. and 70 ms for D.S. Observer I.E. discriminated between T and L with $\text{SOA}(\text{target}) = 120$ ms. For all observers $\text{SOA}(\cdot)$ was 60 ms. *b*, Same as *a*, but discrimination was performed on the central target. Target discrimination rates were 0.90 ± 0.01 , 0.90 ± 0.02 and 0.89 ± 0.02 in *a* and 0.86 ± 0.02 , 0.84 ± 0.02 and 0.83 ± 0.02 in *b* for observers I.E., D.S. and M.S., respectively.

an increase in correct detection for each observer. When examining detection rates for the test flash at different positions in the radial direction, at different eccentricities, a clear enhancement was seen at about 4° eccentricity, where the discrimination target was presented (Fig. 2*a*, left). The same increase in detection rate was seen along the constant eccentricity direction in the vicinity of the discrimination target (Fig. 2*a*, right). To prevent contamination, the test flash was never presented closer than 0.6° from the target centre. To ensure that the increase in detection rate did not originate from trials in which incorrect discriminations were made, we kept the error rate on the discrimination task low by adjusting the masking delay (SOA) for each observer, so that the performance on the discrimination task was $\sim 90\%$ correct (see Fig. 2 legend) for all observers (although one of us served as observer, the other two observers were unaware of the purpose of this study). Surprisingly, the detection rate was lower at positions between the central target (fixation point) and the

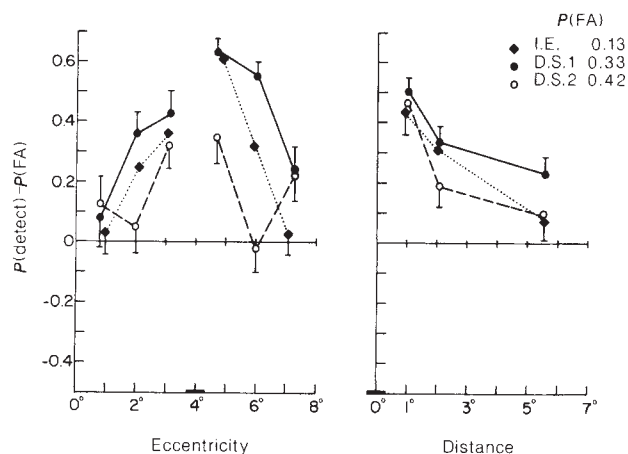


Fig. 3 The same as Fig. 2*a*, but without presentation of the central target. Note that detection at locations between the fixation point and the peripheral target is improved. Data shown are for observers I.E. (T/L discrimination) and D.S. (expt 1, T/L discrimination; expt 2, vertical/horizontal discrimination).

peripheral target than at more peripheral positions. Experiments in which the central target was absent yielded a more symmetrical enhancement around the peripheral target (see Fig. 3). Thus the area of highest detection rate is not around the fixation point, but is shifted to the location of the target being identified; this is demonstrated in a plot of $[P(\text{detect}) - P(\text{FA})]$ isocontours (Fig. 4). The two regions of enhancement were reconstructed from the data of two experiments, a new one with the peripheral target at 2° eccentricity and the one with the peripheral target at 4° , as in Figs 2, 3. The size of the enhancement area is larger at the larger eccentricity, having a diameter about twice that of the enhancement area at 2° eccentricity.

Next we attempted to confirm that this enhancement originated from the discrimination task that our observers were performing, rather than from the presence of the target itself. The presence of the target might cause luminance interaction between itself and the test flash or might bias the observers to detect the test flash when it is located near the target. We performed additional experiments using the same stimuli, but

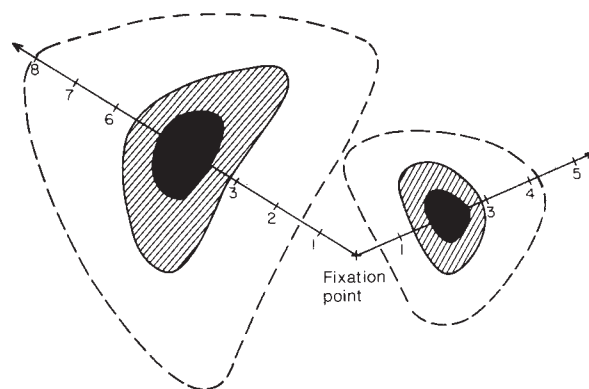


Fig. 4 Test flash detection isocontours plotted at two different eccentricities for observer D.S., in experiments such as those of Fig. 2*a*. The broken lines represent the borders of the enhancement areas where correct detection of the test flash is about the same as without enhancement (Fig. 2*b*), approaching the 'false alarm' rate; within the shaded area the detection rate is more than twice the false alarm rate, and in the solid area about three times the false alarm rate. Note that the linear size of the enhancement area scales linearly with eccentricity, being about twice the size at 4° than at 2° .

asked the observers to perform discrimination on the central target; the results of these experiments are shown in Fig. 2b. The enhancement around the peripheral target disappeared, and performance everywhere was close to chance level [$P(\text{detect}) \sim P(\text{FA})$], with about the same false alarm rates as in the previous experiments). There was also no clear improvement at positions close to the central target. This lack of enhancement might be a result of the coarse sampling used, as the size of the enhancement area around the centre of gaze might be smaller than that at the peripheral positions. This decrease in size would be consistent with that seen with decreasing eccentricity (see Fig. 4).

When attention is focused on the discrimination task, does it cause a change of sensitivity or a change of criterion for detection of the test flash? The psychophysical method we used to investigate this question does not allow a direct answer as it was designed to maximize test-flash position uncertainty and to force attention shifts to the discrimination target. To obtain an answer, we would have to present the test flash at known locations on each block of trials, but that might have allowed the observers to shift their attention to the expected location of the test flash, a strategy we wanted to avoid. As a result, the false alarm responses from all locations were combined, thus it is unknown whether there was an increase in false alarm rate in the vicinity of the discrimination target resulting from the discrimination process. However, the false alarm rates measured using the peripheral target were not significantly larger than those for the central target (FA differences of 0.03 ± 0.06 , -0.12 ± 0.06 , 0.09 ± 0.08 for the three observers; $\pm$ s.e.m.).

We conclude, therefore, that visual discrimination involves a process that increases the probability of detecting a nearby test flash. This increase is not due to the presence of the target to be identified nor is it due to any expectation that the test flash will be found at a particular position, cued by the discrimination target, but results from discriminating the target. At present we cannot decide whether the observed visual enhancement is caused by some sensitivity or criterion change, but we find that this enhancement is involved in a task as simple as discriminating between vertical and horizontal lines. This result indicates that even a simple discrimination task cannot be performed passively by the visual discrimination system. The enhancement might be a property of the serial attentive process that is required for discrimination of orientation⁴. According to our finding, the size of the area processed by the attentive system is scaled with eccentricity, having an average diameter of $\sim 3^\circ$ at 4° eccentricity, and $\sim 1.5^\circ$ at 2° eccentricity. Interestingly, this area is the same for the two tasks that we used—orientation discrimination (vertical versus horizontal) and positional relationship discrimination (T versus L). It is possible that the area of visual enhancement that we have plotted here is the 'searchlight' of attention.

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Phorbol esters block a voltage-sensitive chloride current in hippocampal pyramidal cells

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The importance of second-messenger systems in controlling the excitability of neurones and other cells, through modulation of voltage- and calcium-dependent ionic conductances, has become increasingly clear. Cyclic AMP^{1–3}, acting via protein kinase A^{4–6}, has been identified as the second messenger for several neurotransmitters, and recent studies have suggested that activation of protein kinase C may have similar modulatory actions on neurones^{7–11}. Calcium and potassium currents have so far been shown to be the major ionic conductances modified by kinase activation^{4–11}. We now report that hippocampal pyramidal cells contain a previously undescribed voltage-dependent chloride current which is active at resting potential and is turned off either by membrane depolarization or by activation of protein kinase C by phorbol esters. We propose that this current may reside predominantly in the cell's dendritic membrane and thereby may regulate dendritic excitability.

In 53 out of 55 rat hippocampal pyramidal cells in which K⁺ currents were blocked (see Fig. 1 legend), voltage clamp recording with Cl[−]-containing microelectrodes revealed slow inward current relaxations, in response to hyperpolarizing steps from depolarized holding potentials. These current relaxations appeared to be caused by the turning on of an inward current, as the amplitude of the instantaneous current jump at the beginning of a hyperpolarizing step was smaller than that at the end,

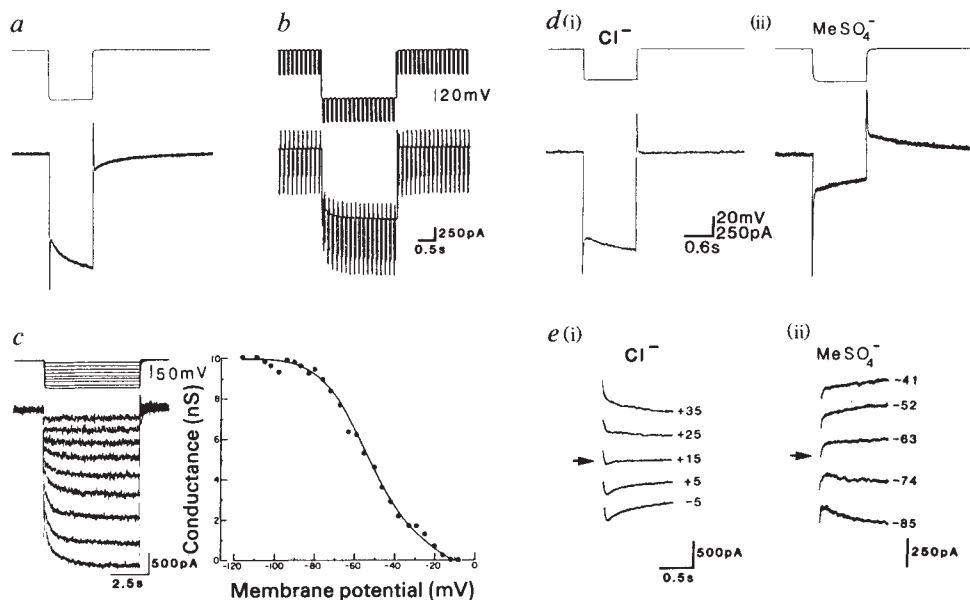
suggesting an increase in membrane conductance during the step (Fig. 1a). In addition, the amplitude of the instantaneous currents produced by short-duration hyperpolarizing voltage steps increased during the inward current relaxation produced by a long hyperpolarizing voltage step, and returned slowly to baseline amplitude when the membrane potential was stepped back to the holding potential (V_H) (Fig. 1b). This is in contrast to the M-current (I_M) recorded in conditions where K⁺ currents are present, which turns off with similar voltage steps¹². Also in contrast to I_M , this current was insensitive to muscarinic receptor activation. This slow inward current was voltage-sensitive over a wide range of membrane potentials (Fig. 1c). As determined by steady-state conductance measurements, the current was completely inactive at membrane potentials positive to approximately -10 mV, was largely active at the resting potential (-55 to -70 mV in these cells), and was fully activated at potentials negative to -90 mV (Fig. 1c). These voltage values could be overestimates of the actual voltage-sensitive range of the current if it was generated at a site electrotonically remote from the recording electrode in the soma (see below). Relaxations caused by this current could also be evoked by holding the cell at relatively hyperpolarized levels and turning it off with depolarizing commands (see Figs 1e, 2b).

We suspected that the ionic carrier of this current was chloride because currents carried by K⁺, Ca²⁺ and Na⁺ should be effectively blocked in the medium used in our experiments (see Fig. 1 legend). Indeed, when cells were impaled with electrodes containing the anion methyl sulphate (MeSO₄[−]), hyperpolarizing steps produced an outward current (Fig. 1d(ii), $n = 11$), rather than an inward current as invariably seen in cells recorded with Cl[−]-filled electrodes (Fig. 1d(i), $n = 53$). The reversal potential of this current was determined with both Cl[−]- and MeSO₄[−]-containing microelectrodes. With the former, when cells were held at hyperpolarized levels and depolarizing steps of increasing size were applied, a turning off of the current was observed, resulting in outward current relaxations which reversed to inward relaxations at a membrane potential of about $+15$ mV in the cell illustrated (Fig. 1e(i)). The average reversal potential of this current with Cl[−] in the recording electrode was

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Fig. 1 A voltage-dependent chloride current ($I_{Cl(V)}$) in hippocampal pyramidal neurones. **a**, A voltage- and time-dependent inward relaxation of membrane current in response to a 1-s hyperpolarizing voltage command from a holding potential (V_H) of -10 mV. An inward tail current follows the return to V_H . This cell was recorded with a CsCl (3 M)-filled microelectrode. **b**, Short-duration hyperpolarizing pulses superimposed on a long-duration hyperpolarizing voltage step show an increase in membrane conductance during the developing inward current relaxation, and a slow return to resting conductance following return to V_H , although little tail current is visible due to the proximity of V_H to E_{Cl} . CsCl-filled microelectrode, $V_H = +5$ mV. **c**, Membrane currents resulting from a family of long-duration hyperpolarizing commands from a V_H of -4 mV. A plot of the



steady-state membrane conductance during the illustrated voltage commands (value plotted is the steady-state conductance during hyperpolarizing commands minus the steady-state conductance at V_H). CsCl-filled microelectrode. **d(i)**, Hyperpolarizing 1-s command from a holding potential of -20 mV in a cell impaled with a KCl-filled microelectrode produces an inward current. **d(ii)**, A similar voltage step in a cell impaled with a KMeSO₄-filled microelectrode produces an outward current; $V_H = -20$ mV. **e(i)**, Tracings of current relaxations produced by depolarizing voltage commands from a holding potential of -50 mV to the voltage indicated at the right of each trace (in mV) in a cell impaled with a KCl-filled microelectrode. **e(ii)**, Tracings of current relaxations produced by hyperpolarizing steps from a holding potential of -21 mV to the voltage (in mV) indicated at the right of each trace in a cell impaled with a KMeSO₄-filled microelectrode. The arrows indicate the approximate value of the reversal potential. Calibrations in **a** are the same as in **d**. Records in **a**, **b** and **d** were photographed from chart records; those in **c** were digitized on a Nicolet 4094 digital oscilloscope and plotted on a Hewlett-Packard 7470A digital plotter; those in **e** were traced by hand from chart records.

Methods. Rat hippocampal slices were prepared and maintained as described previously¹⁹. Intracellular recordings were made from the cell bodies of pyramidal neurones. Microelectrodes were made from thin-wall glass capillaries (Omega Dot; 1.2 mm o.d., 0.9 mm i.d.; Glass Company of America) and contained 3 M CsCl, 3 M KCl or 2 M potassium methyl sulphate (KMeSO₄). Membrane currents were recorded with an Axoclamp-2 single-electrode voltage clamp (Axon Instruments, Burlingame, California). Electrodes were coated to within ~ 50 μ m of the tip with M-coat D (Measurements Group Inc., Raleigh, North Carolina) and the tips were dipped in mineral oil just before use to electrode capacitance. The voltage-clamp head-stage output was monitored continuously and the switching frequency adjusted to the maximum rate, which still allowed the electrode voltage to settle completely between oscillations, usually between 3 and 7 kHz. The superfusing medium used in most experiments was designed to block K⁺, Ca²⁺ and Na⁺ currents and consisted of (mM): NaCl, 70; KCl, 2.5; MgSO₄, 16; NaHCO₃, 26.2; glucose, 11; TEA as chloride, 30; CsCl, 10; and tetrodotoxin (TTX), 0.5 μ M. Our standard medium consisted of (mM): NaCl, 119; KCl, 2.5; CaCl₂, 2.5; MgSO₄, 1.3; NaH₂PO₄, 1; NaHCO₃, 26.2; glucose, 11. Phorbol ester solutions were prepared and bath-applied as described previously⁸.

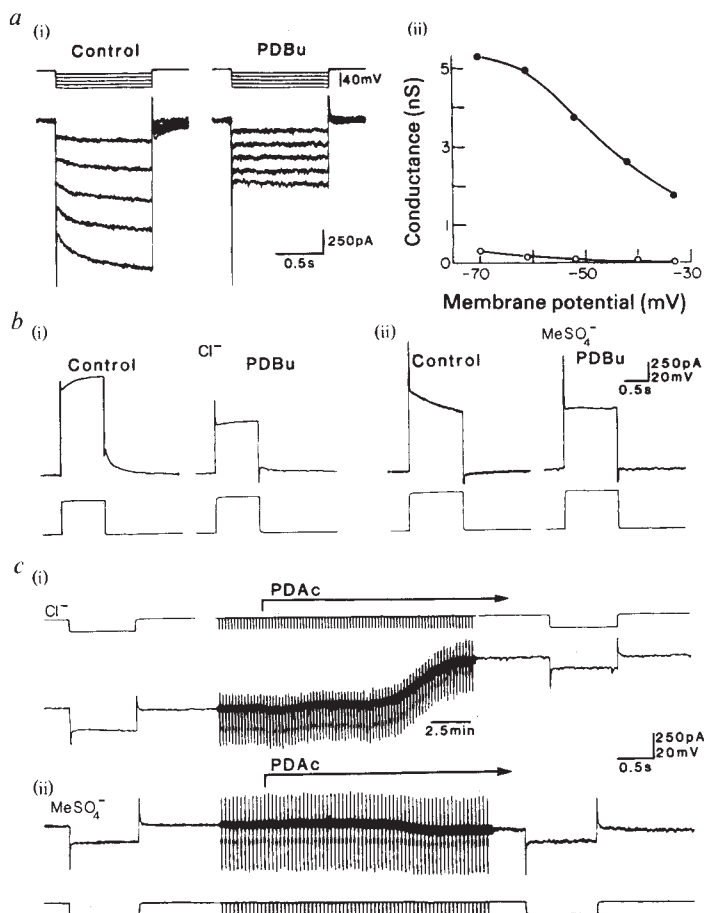
$+9.5 \pm 6.6$ mV (mean $\pm$ s.d., $n = 10$), which is in the range expected for Cl⁻-mediated events in Cl⁻-loaded pyramidal cells¹³. With MeSO₄⁻ in the recording electrode, the outward current produced by hyperpolarizing steps reversed to an inward current at about -70 mV in the cell illustrated (Fig. 1e(ii)), and the average reversal potential was -71 ± 5.2 mV ($n = 8$). The large difference between the reversal potentials in cells recorded with Cl⁻- and MeSO₄⁻-containing electrodes indicates that Cl⁻ ions are the primary charge carrier for this current.

The evidence presented in Fig. 1 indicates that pyramidal cell membranes contain a voltage-sensitive Cl⁻ current that is turned off by depolarization. This current, which we will refer to as $I_{Cl(V)}$, resembles in many respects Cl⁻ currents described in peripheral and invertebrate systems¹⁴⁻¹⁷. However, $I_{Cl(V)}$ differs from a similar current found in *Aplysia* neurones^{15,16} in that it does not require intracellular Cl⁻ loading for its detection. Like the similar voltage-sensitive Cl⁻ current found in rat superior cervical ganglion cells¹⁷, $I_{Cl(V)}$ is blocked by cadmium (100 μ M, $n = 3$), although it is clearly not dependent on Ca²⁺ influx because it is unaffected by medium containing 16 mM Mg²⁺ and no added Ca²⁺ (see Fig. 1).

The application of the phorbol esters 4 β -phorbol-12,13-diacetate (PDAC) or 4 β -phorbol-12,13-dibutyrate (PDBu), known to activate protein kinase C¹⁸, caused a near-total abolition of $I_{Cl(V)}$ at all membrane potentials examined in all 11 cells tested

(Fig. 2a). The blockade occurred regardless of the intracellular anionic composition, as the current relaxations seen with Cl⁻- ($n = 7$; Fig. 2b(i)) or MeSO₄⁻-filled microelectrodes ($n = 4$; Fig. 2b(ii)) were abolished by phorbol esters. When cells were held at -50 mV, a potential at which $I_{Cl(V)}$ is tonically active (see Fig. 1c), the application of phorbol esters to the bathing medium caused opposite shifts in the standing current depending on the anion contained in the microelectrode. When recording with Cl⁻-containing microelectrodes, phorbol esters caused a marked outward shift in standing membrane current which was associated with a decrease in membrane conductance (Fig. 2c(i)), whereas recording with a MeSO₄⁻-containing electrode not only reversed the direction of the $I_{Cl(V)}$ relaxations (Fig. 2b(ii)), but also reversed the direction of the shift in standing current when phorbol ester was applied (Fig. 2c(ii)). This is to be expected because when the microelectrode contains the impermeant MeSO₄⁻, the Cl⁻ current should be in the outward direction rather than inward as it was when Cl⁻ was in the electrode. The small size of the inward shift with MeSO₄⁻ is due to the proximity of the holding potential to the reversal potential of $I_{Cl(V)}$. An analogue of phorbol, 4 α -phorbol-12,13-didecanoate (10 μ M), which has been shown in other systems not to activate protein kinase C¹⁸, had no effect on $I_{Cl(V)}$ ($n = 5$), suggesting that the blockade of $I_{Cl(V)}$ by PDBu and PDAC results from the activation of protein kinase C. The effect of phorbol esters on $I_{Cl(V)}$ is not mediated synaptically, as these effects

Fig. 2 $I_{Cl(V)}$ is blocked by phorbol esters. **a(i)**, A family of currents resulting from hyperpolarizing voltage commands from a V_H of -35 mV in a control and 20 min after application of $10 \mu\text{M}$ 4β -phorbol-12,13-dibutyrate (PDBu). The substantial decrease in resting conductance caused by PDBu was presumably due to the fact that the holding potential of -35 mV was well within the range of $I_{Cl(V)}$ activation (see Fig. 1c); CsCl-filled microelectrode. **a(ii)**, A plot of steady-state membrane conductance against membrane potential corresponding to the traces in **a(i)** (as in Fig. 1c) in control medium (closed circles) and in PDBu-containing medium (open circles). **b**, Depolarizing steps, which turn off $I_{Cl(V)}$, produce outward current relaxations and an outward tail current in cells impaled with CsCl-filled microelectrodes (i) and inward relaxations and tail currents in cells impaled with KMeSO_4 -filled microelectrodes (ii) (V_H in both cases -50 mV). These current relaxations and tail currents were abolished by $10 \mu\text{M}$ PDBu regardless of the recording anion used. **c(i)**, Effects of 4β -phorbol-12,13-diacetate (PDAc) ($10 \mu\text{M}$) on the resting membrane conductance and current at a holding potential of -50 mV in a cell impaled with a KCl-filled microelectrode. In this condition, PDAc caused an outward shift in resting membrane current and a decrease in membrane conductance, as measured by the instantaneous current produced by 10 -mV hyperpolarizing steps. **c(ii)**, Same as **c(i)** except in a cell impaled with a KMeSO_4 -filled microelectrode. In this case PDAc ($10 \mu\text{M}$) caused an inward shift in resting current and a decrease in membrane conductance. The bathing medium for all experiments was the same as for Fig. 1. Records in **a** were digitized on a Nicolet 4094 oscilloscope; **b** and **c** were photographed from chart records.



occur in medium containing no added Ca^{2+} and high (16 mM) Mg^{2+} .

Although we found no direct evidence for the membrane localization of this current, we were unable to detect $I_{Cl(V)}$ in our somatic voltage-clamp recordings in the absence of K^+ -channel blockade. One of several explanations might account for this finding. First, the use of intracellular Cs^+ ions and/or extracellular tetraethylammonium (TEA) or Cs^+ could themselves enhance the size of this current. This seems unlikely as $I_{Cl(V)}$ could be seen clearly after blockade of the M-current and Ca^{2+} -activated K^+ current using bath-applied carbachol ($40 \mu\text{M}$) in otherwise standard medium ($n=4$; not shown). Second, prior blockade of K^+ currents may be necessary for the detection of $I_{Cl(V)}$ because K^+ currents with voltage sensitivities and kinetics similar to $I_{Cl(V)}$ may mask the Cl^- current present in the somatic membrane. $I_{Cl(V)}$ present in the somatic membrane should contribute substantially to somatic resting input resistance because this current is active at membrane potentials near the resting potential. However, $I_{Cl(V)}$ does not seem to contribute significantly to the resting conductance measured at the soma, as phorbol esters do not increase somatic input resistance in standard medium and in fact usually cause a small decrease (ref. 8, but see ref. 7). We favour a third possibility, that a substantial portion of this current is generated at a site electrotonically remote from the soma, perhaps in the dendritic membrane. In this case, blockade of resting K^+ currents would lengthen the space constant of the cell and allow voltage steps imposed at a somatic site to be more effective in eliciting changes in $I_{Cl(V)}$ in the dendrites and would also enhance passive propagation of this current back to the recording site at the soma.

Although a physiological role for $I_{Cl(V)}$ remains to be demon-

strated, it is active at the resting membrane potential, and therefore should contribute substantially to the resting conductance of the membrane at which it is located. If this current is present in dendritic membranes, its blockade by activation of protein kinase C could, by increasing dendritic membrane resistance, increase the transmission of dendritic excitatory events, such as excitatory postsynaptic potentials, to the spike trigger zone in the soma/initial segment.

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Phorbol ester facilitates ^{45}Ca accumulation and catecholamine secretion by nicotine and excess K^+ but not by muscarine in rat adrenal medulla

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Several investigators have shown that tumour promoter phorbol esters mimic the effects of endogenous diacylglycerol to activate a second messenger, protein kinase C (refs 1–3). These phorbol esters have proved to be valuable tools for exploring the role of protein kinase C in many cellular functions^{3–8}. We demonstrate here that secretion of catecholamines evoked from the rat adrenal gland^{9,10} by stimulation of splanchnic nerves, excess potassium (K^+) and nicotine is facilitated by phorbol 12,13-dibutyrate. An inhibitor of protein kinase C, polymyxin B, produced concentration-dependent inhibition of the evoked secretion, and the effect was reversed by the phorbol ester. Furthermore, we show that an increase in the accumulation of radioactively labelled calcium (^{45}Ca) obtained in the adrenal medulla after stimulation with nicotinic agonists and excess K^+ is further enhanced by phorbol ester. Muscarine-evoked secretion of catecholamines, which depends on mobilization of intracellularly bound Ca^{2+} , was not associated with an increase in $^{45}\text{Ca}^{2+}$ uptake, and phorbol ester did not facilitate either catecholamine secretion or $^{45}\text{Ca}^{2+}$ accumulation. We suggest that protein kinase C is involved in the exocytotic secretion of catecholamines by regulating the influx of Ca^{2+} through voltage-sensitive and nicotine receptor-linked Ca^{2+} channels of rat chromaffin cells.

Figure 1 shows that increasing concentrations of phorbol 12,13-dibutyrate (phorbol ester) produce a concentration-dependent increase in catecholamine secretion evoked by stimulation of splanchnic nerves. Secretion increased significantly at 1 nM, more than trebled at 10 nM and was maximal at 30 nM. At 1–30 nM, phorbol ester had no effect on the spontaneous secretion of catecholamines, but 100 nM produced a modest increase in the secretion (16 ± 2 compared with 4 ± 3 ng per 6 min, $n = 12$). The inset of Fig. 1a shows that phorbol ester facilitated the secretion evoked at 1 Hz and 10 Hz; however, the degree of facilitation was significantly ($P < 0.001$) greater at 1 Hz. The most commonly used activator of protein kinase C, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), also enhanced catecholamine secretion evoked at 1 Hz by about fivefold (58 ± 7 compared with 276 ± 31 ng per 6 min, $n = 7$). On the other hand, a non-tumour promoter, phorbol 13-acetate, even in high concentrations (up to 1,000 nM), did not modify catecholamine secretion at rest or after stimulation. The enhancing effect of phorbol ester was reversed within 15 min after the washout, whereas that of TPA lasted for at least 2 h after its washout (not shown).

Several compounds inhibit the activity of protein kinase C isolated from pig heart and rat brain^{12,13}. As shown in Fig. 1b, polymyxin B caused concentration-dependent (10 – $100 \mu\text{g ml}^{-1}$) inhibition of the secretion. The inhibitory effect was fully reversible 30 min after washout of the drug (not shown). Furthermore, prior treatment of the adrenal medulla with 50 nM phorbol ester partially reversed the inhibitory effects of $100 \mu\text{g ml}^{-1}$ polymyxin B, almost prevented the inhibitory effects of $30 \mu\text{g ml}^{-1}$ polymyxin B, and produced a significant enhancement in the presence of $10 \mu\text{g ml}^{-1}$ polymyxin B (Fig. 1b).

The increased catecholamine secretion described in Fig. 1a could be a result of the action of phorbol ester either on splanchnic nerves to release more acetylcholine or on postsynaptic

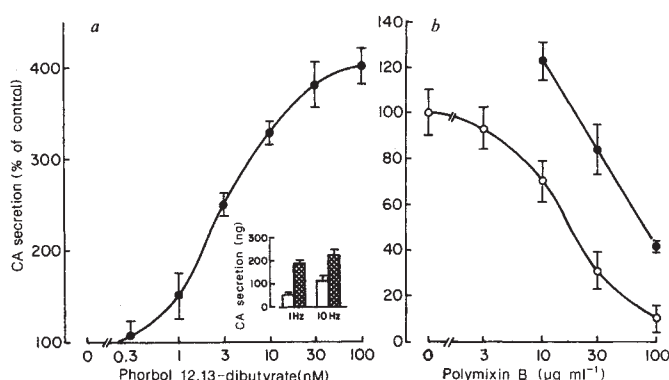


Fig. 1 a, Facilitation by phorbol 12,13-dibutyrate of the secretion of catecholamines (CA) evoked by stimulation of splanchnic nerves. b, Polymyxin B-induced inhibition of catecholamine secretion evoked by nerve stimulation, and its reversal by phorbol ester. In a, the left adrenal glands of male rats (300–350 g) were prepared for *in vitro* perfusion as described previously⁹. The perfusion medium was Krebs–bicarbonate solution and the rate of perfusion was 0.35 ml min^{-1} at 37°C . Ag/AgCl electrodes were used for transmural stimulation of the gland, and the parameters were set to stimulate selectively presynaptic splanchnic nerve terminals^{9,10}. Splanchnic nerves were stimulated at 1 Hz (300 pulses), first without phorbol ester and then in increasing concentrations of phorbol ester (●). Each concentration remained in contact with the adrenal medulla for 15 min before collection of samples. Perfusate was collected for 6 min to determine catecholamine secretion in the absence of stimulation, and then for another 6 min, during which the gland was stimulated for 300 s. Amounts of catecholamines secreted during the immediately non-stimulation period have been subtracted from those secreted during stimulation period to obtain net catecholamine secretion, which is expressed either in per cent or in absolute values. In a all values are expressed as per cent of the control, which was $64 \pm 6 \text{ ng}$, $n = 16$. The inset shows the effect of 30 nM phorbol ester on catecholamine secretion evoked by delivering 300 pulses at 1 and 10 Hz. Open columns, control; cross-hatched columns, phorbol ester–Krebs solution. Catecholamine content of the perfusate was measured directly by the fluorometric method as described elsewhere^{9,11}. Adrenaline (base) was used as a standard; all values are presented as means with standard errors, and differences were compared using Student's paired *t*-test. Phorbol ester (LC Services, Woburn, Massachusetts) was dissolved in dimethyl sulphoxide and added to Krebs solution. The final dilution of the solvent in the Krebs solution was 500–100-fold. At these dilutions, the solvent alone had no effect on catecholamine secretion when added to Krebs solution. Each dot and column is a mean of four experiments. Vertical lines show s.e.m. In b, catecholamine secretion was evoked by stimulation of splanchnic nerves at 10 Hz (300 pulses), first in Krebs solution and then in increasing concentrations of polymyxin B (○). The adrenal medulla was pretreated with each concentration for 15 min, and then nerves were stimulated in the presence of the agent. After testing the highest concentration, the adrenal gland was perfused with Krebs solution for 30 min to determine whether the inhibitory effect was reversible. In all the glands tested ($n = 5$), secretion was restored to $110 \pm 14\%$ of the initial control value. The perfusion medium was then changed over to 50 nM phorbol ester–Krebs solution for 10 min, and in its presence various concentrations of polymyxin B were added (●) for 15 min and then samples (spontaneous and stimulated) collected. All values are expressed as per cent of control, which was $103 \pm 9 \text{ ng}$ ($n = 7$). The entire protocol was carried out in the same adrenal gland. Each point, except the control, represents a mean of four experiments. Vertical lines show s.e.m.

chromaffin cells to release more catecholamine from acetylcholine-induced activation of the nicotinic and muscarinic receptors^{9,10}. To distinguish between these possibilities, we investigated the effect of phorbol ester on catecholamine secretion evoked by direct stimulation of chromaffin cells with cholinergic agonists as well as excess K^+ (Fig. 2).

Secretion of catecholamines evoked by nicotine, carbamylcholine and excess K^+ was approximately doubled by 30 nM phorbol ester. However, secretion evoked by muscarine, which was of the same order of magnitude as that evoked by nicotine, remained unaffected by phorbol ester. Because of these differential effects of phorbol ester, it was essential to know the role of Ca^{2+} in the secretion evoked by nicotine and muscarine. Perfusion of the adrenal gland with Ca-free and 1 mM EGTA–Krebs solution for 15 min completely blocked the secretion evoked by nicotine and muscarine (Fig. 3), and addition of phorbol ester to this medium had no effect on secretion evoked by either agent. If the same adrenal gland was perfused with 0.1 mM

Table 1 Effects of different stimulatory agents on $^{45}\text{Ca}^{2+}$ accumulation and secretion of catecholamine in absence and presence of phorbol ester

Stimulating agent	Catecholamine secretion (ng mg $^{-1}$)		$^{45}\text{Ca}^{2+}$ accumulation (pg mg $^{-1}$)	
	Krebs	Phorbol ester* (30 nM)	Krebs	Phorbol ester* (30 nM)
None (control)	2 $\pm$ 0.4	2 $\pm$ 0.4 (NS)	110 $\pm$ 20	123 $\pm$ 25 (NS)
Muscarine (30 $\mu\text{g ml}^{-1}$)	38 $\pm$ 4.5	41 $\pm$ 5.1 (NS)	126 $\pm$ 11	124 $\pm$ 28 (NS)
Carbamylcholine (4 $\mu\text{g ml}^{-1}$)	39 $\pm$ 6.3	68 $\pm$ 7.4 ($P < 0.001$)	303 $\pm$ 34	489 $\pm$ 32 ($P < 0.001$)
Nicotine (3 $\mu\text{g ml}^{-1}$)	29 $\pm$ 0.2	67 $\pm$ 8.2 ($P < 0.001$)	343 $\pm$ 21	463 $\pm$ 26 ($P < 0.001$)
Excess K $^{+}$ (1.5 mg ml $^{-1}$)	48 $\pm$ 6.5	91 $\pm$ 17.4 ($P < 0.001$)	303 $\pm$ 25	507 $\pm$ 48 ($P < 0.001$)

Thirty minutes after perfusion of the adrenal gland with Krebs solution, the medium was changed to Krebs solution containing 0.5 $\mu\text{g ml}^{-1}$ $^{45}\text{Ca}^{2+}$ (as $^{45}\text{CaCl}_2$, specific activity 23.69 mCi mg $^{-1}$; NEN) for 10 min. The gland was perfused for a further 30 min with Krebs solution to wash out most of the extracellular $^{45}\text{Ca}^{2+}$. Within this time period the radioactivity in the wash fluid approaches a very low and steady-state level ($\sim 2\%$ of that in the first 5-min period). An identical protocol was then carried out in further series of adrenal glands, except that the 10-min perfusion with $^{45}\text{Ca}^{2+}$ -Krebs solution contained various secretagogues. Perfusates collected for 10 min during $^{45}\text{Ca}^{2+}$ exposure and stimulatory agents were assayed for catecholamine to ensure the expected effects of these agents on catecholamine secretion. NS, statistically non-significant; other P values show comparison between the data obtained in Krebs solution and in phorbol ester-Krebs solution.

* In another series, the above protocol was carried out, except that before exposure to $^{45}\text{Ca}^{2+}$ medium the adrenal gland was perfused with Krebs solution containing 30 nM phorbol ester for 15 min. Phorbol ester was also present during 10 min perfusion with $^{45}\text{Ca}^{2+}$ medium alone, or that containing stimulatory agent. After the 30-min wash period, the adrenal gland was removed, and the adrenal medulla separated, weighed, homogenized in 1 ml of 0.05 M perchloric acid; the homogenate was centrifuged and the supernatant (0.5 ml) was counted in a liquid scintillation counter (Beckman, model LS7000). Total counts after correction for dilution and quenching were converted to pg and expressed as pg per mg of the wet weight of the adrenal medulla. Each value represents a mean of four or five experiments.

Ca-Krebs solution and the secretion was evoked by both agents, nicotine-evoked secretion was $<20\%$ of that in 2.5 mM Ca medium, but that evoked by muscarine was $\sim 65\%$ of the control value. Phorbol ester increased nicotine-evoked secretion by more than 300% in 0.1 mM Ca medium, but muscarine-evoked secretion was enhanced only 15% in the same medium (statistically not significant, $P > 0.5$).

The effect of phorbol ester on $^{45}\text{Ca}^{2+}$ accumulation in the adrenal medulla was investigated at rest and after stimulation with different agonists (Table 1). Although muscarine increased catecholamine secretion from a basal level of 2 ng to 38 ng, it was not accompanied by a significant increase in the accumulation of $^{45}\text{Ca}^{2+}$ over the control value. More importantly, phorbol ester did not enhance the effect of muscarine on catecholamine secretion and $^{45}\text{Ca}^{2+}$ accumulation. Catecholamine secretion evoked by carbamylcholine and nicotine, which was almost identical to that evoked by muscarine, was associated with a marked increase (275%) in $^{45}\text{Ca}^{2+}$ accumulation. In the presence of phorbol ester, the secretion was even further enhanced, and that too was associated with an additional increase (400%) in $^{45}\text{Ca}^{2+}$ uptake. K $^{+}$ -evoked secretion was also accompanied by a large increase in the accumulation of $^{45}\text{Ca}^{2+}$ and was further enhanced by the phorbol ester. Catecholamine secretion and $^{45}\text{Ca}^{2+}$ accumulation under resting conditions were not affected by the phorbol ester.

Phorbol 12,13-dibutyrate significantly enhanced catecholamine secretion evoked by several procedures that activate chromaffin cells, either directly or indirectly, through the splanchnic nerves. We believe this to be the first report in which phorbol ester has produced a clear-cut facilitation of catecholamine secretion after activation of nicotinic receptors and depolarization of chromaffin cells. Furthermore, we show that an inhibitor of protein kinase C, polymixin B (ref. 13), almost completely inhibits catecholamine secretion, and the effect is reversed by the phorbol ester. This is the first example in which phorbol ester was able to overcome the inhibitory effects of an inactivator of protein kinase C to restore the secretory process. Assuming that the major target of action of phorbol ester and polymixin B in the rat chromaffin cells is the protein kinase C, it is tempting to postulate that this second messenger is involved in the exocytotic secretion of adrenal medullary hormones. Knight and Baker first studied the effects of phorbol ester on catecholamine secretion in bovine adrenal medullary cells, and found that Ca^{2+} sensitivity of the secretory process was enhanced by TPA. They therefore concluded that protein kinase C is involved in exocytosis⁴. Other investigators

have reached a similar conclusion by studying the effects of phorbol ester in cultured bovine chromaffin cells and pheochromocytoma (PC12) cells¹⁴⁻¹⁶. However, in none of these experiments was it possible to show facilitation by phorbol ester of catecholamine secretion evoked by a nicotinic agonist or excess K $^{+}$.

The next question is at which step protein kinase C participates in the process of exocytosis. We have found that the net increase in $^{45}\text{Ca}^{2+}$ accumulation obtained after stimulation of chromaffin cells with nicotinic agents and excess K $^{+}$ was further enhanced by phorbol ester. We therefore propose that protein kinase C may actually control the transport of Ca^{2+} entering through nicotinic receptor-operated and voltage-sensitive Ca^{2+} channels of the chromaffin cells. Recently, it has been reported that

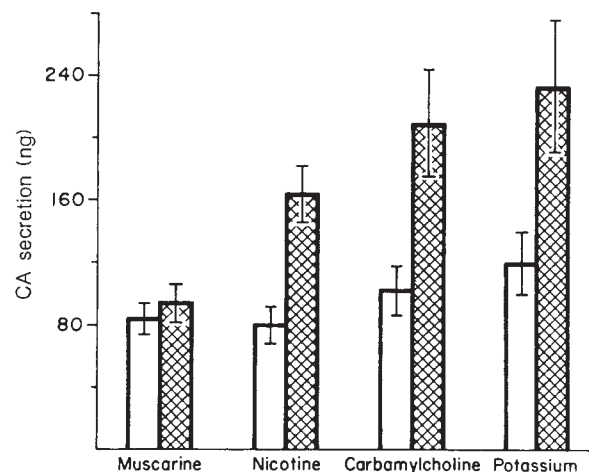


Fig. 2 Effects of phorbol ester on catecholamine secretion evoked by nicotinic agonists, muscarine and excess K $^{+}$. Adrenal gland was perfused with Krebs solution and secretion was then evoked by injecting muscarine (30 μg) and nicotine (0.3 μg) (open columns) within 20 min of each other. The perfusion medium was then changed to Krebs solution containing 30 nM phorbol ester for 15 min and in its presence muscarine and nicotine were injected (cross-hatched columns), as described above. The same protocol was carried out in another series of adrenal glands, except that nicotine was replaced with either carbamylcholine (4 μg) or KCl (40 μg) before (open columns) and after (cross-hatched columns) 30 nM phorbol ester. All agents were injected in a total volume of 100 μl saline. Each column is a mean of 5 experiments, except the column for muscarine, which is a mean of 10 experiments.

Vertical lines represent s.e.m.

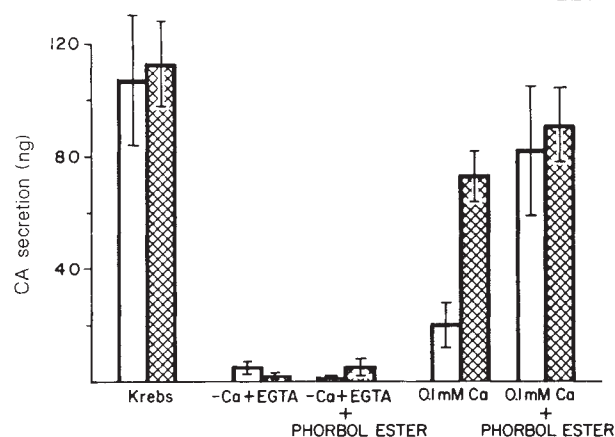


Fig. 3 Role of Ca^{2+} in the enhancement of catecholamine secretion evoked by nicotine and muscarine. Secretion was evoked by nicotine (open column) and muscarine (cross-hatched column), as described in Fig. 2 legend, in Krebs solution, Ca^{2+} -free plus 1 mM EGTA-Krebs solution and 0.1 mM Ca^{2+} -Krebs solution, before and after addition of 30 nM phorbol ester to various solutions. The adrenal gland was perfused with each solution for 15–30 min before sample collection. The entire protocol was carried out in the same adrenal gland. Each column represents a mean of four experiments. Vertical lines represent s.e.m.

injection of purified protein kinase C into *Aplysia* neurones or treatment with phorbol ester enhances the potential-sensitive membrane Ca^{2+} current¹⁷. It is likely that protein kinase C regulates the activity of Ca^{2+} channels by phosphorylating membrane proteins involved in opening and closing these channels. Protein kinase C-induced phosphorylation of different types of proteins associated with cytosol, cytoskeleton and other unidentified cell organelles of neuronal tissues has been documented^{18–22}. Our present work shows that phorbol ester alone does not increase catecholamine secretion and has no effect on $^{45}\text{Ca}^{2+}$ accumulation. This means that active protein kinase C itself cannot affect the kinetics of Ca^{2+} channel opening and that their opening depends on other intracellular messengers generated by nicotinic receptor activation and membrane depolarization.

Another important finding leading to our proposal that protein kinase C regulates Ca^{2+} channels was the inability of phorbol ester to increase muscarine-evoked catecholamine secretion and of muscarine to increase $^{45}\text{Ca}^{2+}$ accumulation in either the absence or presence of phorbol ester. Note that Ca^{2+} is essential for muscarine-evoked secretion. These results clearly demonstrate the differential modes of Ca^{2+} mobilization by nicotinic and muscarinic agonists in the rat chromaffin cells; nicotine enhances the influx of extracellular Ca^{2+} , whereas muscarine mobilizes intracellularly bound Ca^{2+} . In bovine adrenal medullary cells, muscarine produces a modest rise in intracellular Ca^{2+} that is independent of external Ca^{2+} (ref. 23), however this increase in Ca^{2+} concentration does not lead to catecholamine secretion²⁴. In the rat chromaffin cell, muscarine must mobilize large amounts of intracellular Ca^{2+} to raise its levels sufficiently high to initiate secretion. Because muscarine-evoked secretion is unaffected by phorbol ester, it is likely that the role of protein kinase C is restricted to the events regulating the influx of extracellular Ca^{2+} rather than those affecting the steps involved in the utilization of Ca^{2+} in exocytosis.

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Mediation of mouse natural cytotoxic activity by tumour necrosis factor

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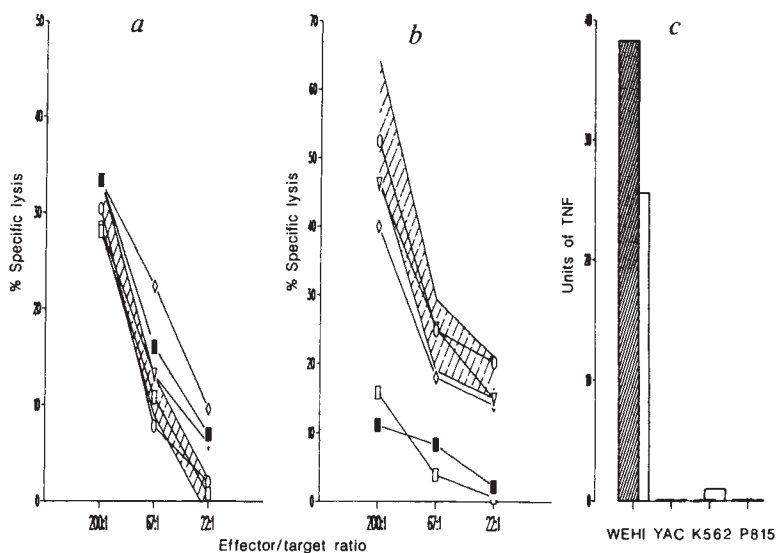
Natural cell-mediated cytotoxic activity in the mouse has been associated with two types of effector cells, the natural killer (NK) cell and the natural cytotoxic (NC) cell, which seem to differ with regard to their patterns of target selectivity, cell surface characteristics and susceptibility to regulatory factors¹. During studies on the mechanism of action of cytotoxic molecules, it became evident that WEHI-164, the prototype NC target cell, was highly susceptible to direct lysis by both human and mouse recombinant tumour necrosis factor (TNF). Here we show that NC, but not NK activity mediated by normal splenocytes, is abrogated by rabbit antibodies to recombinant and natural TNF, respectively. Thus, the cell-mediated activity defined as NC is due to release of TNF by normal spleen cells and does not represent a unique natural effector mechanism.

The prototype target for detection of mouse NK activity is the YAC-1 lymphoma cell line², whereas WEHI-164 cells are the main targets used to selectively measure NC activity^{1,3}. Mouse NK cells have been shown to express asialo-GM₁, and the alloantigens of Ly5, Qa5, NK1 and NK2, on the cell surface^{2,4,5}. Some mouse NK cells also express Thy-1 antigen. In contrast, treatment of cells bearing any of these markers with antibody plus complement has little effect on NC activity^{1,3,6}. NK activity is quite rapid, with close to maximum levels achieved within 4 h, whereas NC activity usually requires incubation periods of 18 h or longer^{1,5}. NK activity has been shown to be strongly augmented by pretreatment of the effector cells with interferon or interleukin-2 (refs 5, 7, 8), whereas NC activity has been shown to be enhanced primarily by interleukin-3 (refs 7, 9). Mouse NK activity, analogous to previous findings with human and rat NK cells⁵, has been closely associated with a morphological counterpart, the large granular lymphocyte⁵. In contrast, it has proved difficult to directly associate NC activity with any specific cell type^{5,9}, raising the possibility that this activity is not a direct cell-mediated event. In addition to the

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Fig. 1 *a, b*, Effect of antisera or monoclonal antibodies on cell-mediated lysis of YAC-1 (NK) and WEHI-164 (NC) cells, respectively. *c*, TNF stimulation by various target cells. The per cent specific lysis of ^{51}Cr -labelled WEHI-164 and YAC-1 targets in an 18-h or 4-h assay^{8,9} is shown in *a* and *b*, respectively. Effector cells from 7-week-old C57BL/6 mice were tested at various effector/target cell ratios (200:1, 67:1 and 22:1, with 5,000 targets per well) in a standard microcytotoxicity assay⁸. The specific lysis above that obtained in control medium is shown. The shaded area represents the control level of cytotoxicity (mean $\pm$ s.d.) mediated by untreated spleen cells. A final 1:40 dilution of each antibody was added to the assay. Similar results were seen with a 1:200 final dilution, with approximately 50% inhibition of cytotoxicity seen with antisera to mouse TNF (data not shown). ■, Rabbit anti-TNF-1 antiserum (300 TNF neutralizing units); □, rabbit anti-TNF-2 antiserum (300 TNF neutralizing units); ○, monoclonal anti-human TNF antibody (1,000 TNF neutralizing units); ▽, monoclonal anti-human LT antibody (1,000 LT neutralizing units); ◇, normal rabbit serum (obtained from a pre-bleed for anti-TNF-1 antiserum). Similar results to those for NRS were seen with two other normal rabbit sera (data not shown). *c*, Plot of units per ml of TNF produced during co-culture of nylon-passed C57BL/6 spleen cells (2×10^6) with various targets: NK (K562-human NK), NC (WEHI) or NK/NC-insusceptible (P815). Supernatants from reactions with effector splenocytes/target cell ratios of 200:1 (■) or 50:1 (□) were collected after 18 h, filtered and units of mouse TNF determined relative to a recombinant mouse TNF standard curve. Controls consisting of cells alone and targets alone yielded no detectable TNF production (data not shown).



incomplete characterization of the NC effector cells, there is also little information available concerning the mechanism of cytotoxicity of NC cells. In contrast, recent studies have indicated that a highly cytolytic protein in the granules of large granular lymphocytes has a key role in the cytotoxic activity of NK cells¹⁰. Also, NK cells have been shown to release a soluble cytotoxic factor (NKCF) on interaction with NK-susceptible target cells¹¹. Thus, it was of considerable interest to determine whether a similar mechanism of cytotoxicity was used by NC cells.

During re-examination of the spectrum of cytotoxic activity reported for human tumour necrosis factor (TNF)¹², we found that WEHI-164 cells were highly susceptible to the effects of this cytotoxic factor, which raised questions regarding the possible role of TNF in the mechanism of cytotoxicity of NC cells. We have found that WEHI-164 cells are highly susceptible to

both recombinant mouse TNF and recombinant human TNF. In addition, NC activity by mouse spleen cells was strongly inhibited by two independently produced rabbit antisera raised against mouse TNF. Thus, the cell-mediated cytotoxicity defined as NC activity appears to be attributable to release of TNF *in vitro* by normal mouse spleen cells. These data also indicate that this cytokine may be produced spontaneously by certain normal cells and thereby contribute to natural cell-mediated cytotoxicity.

Table 1 shows the susceptibility of WEHI-164 cells to lysis by purified recombinant mouse TNF (Biogen SA) and by recombinant human TNF¹³ (provided by Dr M. Shepard, Genentech, Inc., South San Francisco). Both recombinant TNFs had cytolytic activity against WEHI-164 cells but had little effect on YAC-1, the classical mouse target cells for NK activity. To confirm that the activities observed were indeed due to TNF,

Table 1 Effect of anti-TNF and anti-LT antibodies on lysis of WEHI-16 cells by recombinant mouse or human TNF

% Specific lysis in TNF in the presence of various antibodies							
WEHI-164							
Agent (U ml ⁻¹)	YAC-1	None	Anti-TNF-1	Anti-TNF-2	Monoclonal anti-TNF	Monoclonal anti-LT	NRS
MuTNF (500)	2.2	68.8	13.3	36.8	78.5	80.4	76.8
MuTNF (50)	2.1	54.6	11.6	33.7	62.5	57.5	52.5
MuTNF (5)	0.5	41.4	NT	NT	NT	NT	NT
HuTNF (500)	3.2	65.3	NT	NT	10.2	68.7	NT
HuTNF (50)	-1.1	56.7	NT	NT	5.4	54.3	NT
HuTNF (5)	0.1	44.3	NT	NT	1.3	40.3	NT

The per cent specific lysis of ^{51}Cr -labelled WEHI-164 target cells in an 18-h release assay is shown^{8,9}. The results presented indicate the per cent release of ^{51}Cr above the background of spontaneous release in medium alone. The table shows the effects of rabbit anti-mouse TNF-1 (300 TNF neutralizing units), rabbit anti-mouse TNF-2 (300 TNF neutralizing units), monoclonal anti-human TNF antibody (1,000 TNF neutralizing units), monoclonal anti-human LT antibody (1,000 LT neutralizing units), and normal rabbit serum (NRS) obtained from a preimmunization bleeding of the rabbit used for production of anti-TNF-1. The assay was performed as described previously^{8,9}. Recombinant human (Hu) and mouse (Mu) TNF were purified from *Escherichia coli* cells containing HuTNF and MuTNF gene-encoded plasmids. The cells were broken using a Manton-Gaulin press. The TNF in the supernatant was then purified using DEAE-Sepharose CL6B, phenyl-Sepharose CL4B and Sephacryl S-200. The proteins were purified to greater than 97% purity with a specific activity of 3×10^7 U mg⁻¹ for MuTNF and 1.5×10^7 U mg⁻¹ for HuTNF. One unit of TNF, as defined previously¹², is the amount resulting in 50% inhibition of growth of the L929 cell line. Anti-MuTNF antiserum was produced in a New Zealand White female rabbit by intradermal immunization at multiple sites with a suspension of purified mouse TNF (200 $\mu\text{g ml}^{-1}$ in phosphate-buffered saline, 1 ml) in Freund's complete adjuvant (1 ml); this was followed by a booster injection at 1 month. Serum was obtained 10-14 days after the boost. The rabbit produced satisfactory titres of antibody (<3,000) as determined by enzyme-linked immunosorbent assay. Various concentrations of recombinant mouse or human TNF preparations were added to labelled target cells in the presence of various concentrations of antisera or monoclonal antibodies. A final 1:40 dilution of each antibody was added to the assay. NT, not tested.

we determined the effects of specific anti-TNF antibodies. The cytotoxicity of recombinant mouse TNF for WEHI-164 target cells was inhibited strongly by two different rabbit antisera, raised independently against either the purified natural mouse cytokine (anti-TNF-1) or recombinant mouse TNF (anti-TNF-2). In contrast, monoclonal antibody to human TNF or lymphotoxin (LT), or normal rabbit serum (NRS), had no significant effect on the cytotoxicity of recombinant mouse TNF. However, monoclonal antibody to human TNF blocked human TNF-mediated lysis of WEHI-164 cells (Table 1). The species specificity of this monoclonal antibody suggests that the blocking effect is not dependent on the NC target, but rather on the source of TNF. It is noteworthy that YAC-1 cells were highly resistant to lysis by either human or mouse TNF. Addition of antisera against target cells had no toxic effect on NK or NC targets (data not shown).

To study more directly the possible involvement of TNF in NC activity, the antibodies to mouse TNF were added to a standard NC assay (Fig. 1). Each of these antibodies to murine TNF strongly inhibited the lysis of WEHI-164 cells by normal mouse spleen cells. Based on calculations of the lytic units, derived from the cytotoxicity data at several effector to target cell ratios, greater than 90% of the lytic activity was neutralized by each of the rabbit anti-mouse TNF antibodies. In contrast, no significant inhibition was seen with the NRS controls including sera from preimmunization bleeds, or with monoclonal antibody to human TNF or LT. Parallel experiments were performed to evaluate the possible effects of the antibodies on mouse NK activity against YAC-1 target cells. No significant inhibition of NK lysis was seen with any of the antibodies (Fig. 1). The increase in NK lysis seen at low effector/target cell ratio with selected antisera was not observed in all experiments or at higher dilutions of antisera. Thus, two antisera to mouse TNF (one raised against recombinant and the other against highly purified natural mouse TNF) selectively inhibited cell-mediated cytotoxicity against the NC target cells but had no effect on NK activity. This result further demonstrates a major distinction between these two forms of natural cytotoxicity.

We have demonstrated that NC activity against WEHI-164 target cells appears to be a cytokine-mediated phenomenon, apparently mediated by the spontaneous release of TNF during the cytotoxicity assay. The mediation of NC activity by the release of TNF may explain the prolonged incubation periods required to demonstrate this activity, in contrast to the rapid kinetics of NK activity. This notion is supported by the results in Fig. 1c, which demonstrate high levels of TNF production only in co-cultures with WEHI-164, but not in co-cultures with NK or other potential targets. Presumably the extended period of incubation is required for sufficient release, if not also production, of the cytokine. The association of TNF with NC activity also provides new clues as to the nature of the NC cell. TNF has been found to be produced predominantly by macrophages^{12,15}. The possible derivation of NC cells from the myelomonocytic lineage would be consistent with previous evidence for adherence properties of NC cells^{1,5,9}, the resistance of NC activity to treatments which strongly inhibit lymphocyte numbers and functions (for example, a high dose of cyclophosphamide and anti-lymphocyte antibodies plus complement), and the ability of interleukin-3, a cytokine with multipotential colony-stimulating factor effects on monocytic cells as well as other haematopoietic cells, to promote both the growth and cytolytic activity of NC cells^{5,7,9}.

Our observation that TNF has a key role in NC, but not NK, activity raises the possibility of a new approach to discriminating between NC and NK activities. Although it was initially thought that most solid tumour cells were selectively sensitive to NC cells and that lymphoma cells were preferentially lysed by NK cells⁹, it has recently been found that many target cells of both types are susceptible, to a certain extent, to lysis by each category of effector cells⁵. Monoclonal antibody to TNF

may help to determine the contribution of NC cells to the natural cell-mediated lysis of various target cells. Also, in analogy to the present results with mouse NC cells, it will be of considerable interest to study the effects of antibodies to human TNF on the recently reported human counterpart to NC activity¹⁴.

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A block to elongation is largely responsible for decreased transcription of *c-myc* in differentiated HL60 cells

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The *c-myc* gene product is a nuclear protein^{1,2} expressed in a wide variety of cell types³. It has been implicated in the control of normal cell growth as well as transformation⁴⁻⁷, but its exact function is unknown. When the human promyelocytic leukaemia cell line HL60 is treated with retinoic acid, the cells differentiate into granulocytes, and there is a reduction in steady state *c-myc* RNA of more than 10-fold⁸. Nuclear runoff assays show that this reduction is caused by a corresponding decrease in the transcription of exon 2. However, only a minor decrease in exon 1 transcription is observed upon differentiation. In undifferentiated HL60 cells there is an approximately 3-fold molar excess of exon 1 transcription over exon 2, and this excess increases to about 15-fold in differentiated cells. This observation suggests that a major component of *c-myc* transcriptional down-regulation in HL60 cells is at the level of elongation rather than at the level of initiation. The position of the elongation block was mapped to the region of the boundary between exon 1 and intron 1. During HL60 differentiation, a DNase I hypersensitive site in the chromatin about 300 bases downstream of the 5' end of intron 1 increases in intensity relative to other sites, possibly reflecting events associated with the termination of transcription. Our runoff analysis also revealed transcription of both strands immediately upstream of exon 1 in HL60 cells. The sense strand transcription of this region produces a novel *c-myc* RNA which initiates several hundred bases upstream of the previously defined promoters and is found in a variety of cell types.

Large changes in *c-myc* RNA levels occur during terminal differentiation^{8,9} and mitogenic stimulation of quiescent cells⁵. Both transcriptional¹⁰ and post-transcriptional mechanisms¹¹ have been reported to control the transient increase of *c-myc* expression which follows mitogenic stimulation. In contrast, post-transcriptional destabilization alone has been reported to account for the reduction in *c-myc* RNA level of differentiating

Fig. 1 Runoff transcription of *c-myc* in HL60. **Steady state RNA:** Slot blotted total RNA (4 μ g) from the same cell prep used for the run-off assays shown to the left was hybridized to a 32 P-RNA exon 3 probe. By Northern analysis, this probe detects only genuine *c-myc* transcripts. **A,** Hybridization of RNA (15 $\times$ 10⁶ c.p.m.) from HL60 uninduced or induced for 2.5 days in retinoic acid (1 μ M). Probes marked +, detect sense transcripts and - detect anti-sense transcripts. As controls equimolar amounts of the p ϵ 0.7 ϵ -globin plasmid¹⁸ and the granulocyte specific Φ 11 cDNA clone (1.4 kb) were used. **α -amanitin:** Hybridization of RNA from uninduced HL60 synthesized in the presence of 2 μ g ml⁻¹ α -amanitin. The number of cells used was 33% more than in untreated samples. Under these conditions the amount of rRNA synthesized is as great as in untreated samples (not shown). **SP6:** Hybridization of SP6 RNA from a full length P0 cDNA clone¹⁹. Transcripts were eluted from a gel and degraded in NaOH prior to hybridization. **Sarkosyl:** Hybridization of RNA from uninduced HL60 synthesized in the presence of 0.5% sarkosyl. **Manca:** Run-off transcripts from the Manca, B-cell lymphoma. **B,** Map of the restriction fragment probes, a-d, cloned in M13 vectors and summary of *c-myc* transcription pattern in HL60. The mapping of the P0 promoter will be published elsewhere¹⁹. The lengths of the probes are: a, 586, b, 535, c, 443, d, 414 bases. They were subcloned from the genomic clone pMC41-HE²⁰ (a) or the P0 cDNA clone (b-d).

Methods. Preparation of nuclei was as described (15) and equivalent results were obtained from fresh and frozen nuclei. The results shown except for Manca were from nuclei pretreated with 10 μ g ml⁻¹ RNase A for 30 min on ice¹⁶ but identical results were also obtained with untreated nuclei. (In the latter case 32 P-RNA was hydrolysed with 0.3 M NaOH for 10 min on ice.) RNase was inactivated with RNasin (200 U ml⁻¹) and washing once in 500 μ l nuclear freezing buffer (NFB; ref. 15) plus RNasin. The nuclei ($\approx 3 \times 10^7$) were then resuspended in 210 μ l of NFB and the runoff reaction performed as in ref. 17. The 32 P-RNA was on average 100 bases long. Hybridizations to 0.5 μ g of single-stranded M13 DNA slot blotted in 20 $\times$ SSC were carried out at 0.3 M NaCl and 65 $^{\circ}$ C for 36-40 h followed by washing in 0.1 $\times$ SSC, 0.1% SDS at 65 $^{\circ}$ C (ref. 17). Washing the filters at 72 $^{\circ}$ C did not alter the signal ratio of the *c-myc* probes. Autoradiographs were exposed for 2 days.

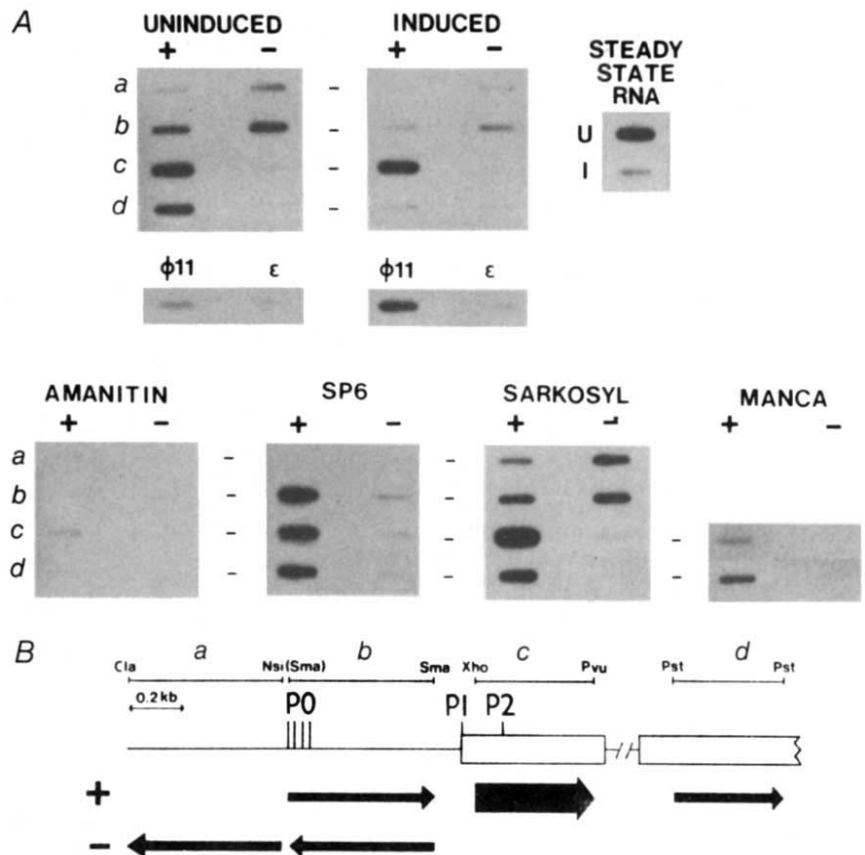
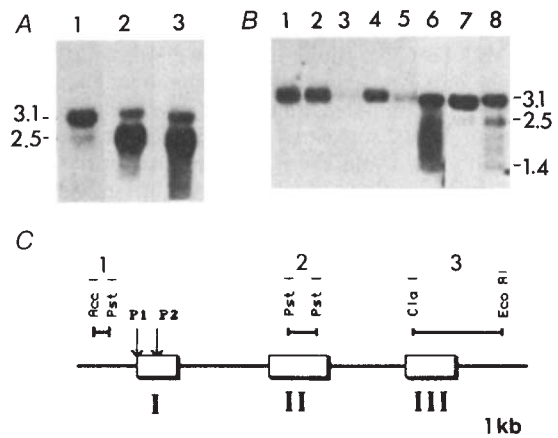


Fig. 2 **A,** Northern blot of *c-myc* RNAs in HL60 cells. Lanes 1-3: Poly (A)⁺ RNA (10 μ g) was analysed by hybridization to the corresponding nick-translated restriction fragments in Fig. 1c. Autoradiography was for 20 h, 1 h, and 1 h for lanes 1, 2 and 3 respectively. **B,** P0 transcripts in different cell types. Northern filters containing poly(A)⁺ RNA (1-5 μ g) were hybridized to an anti-sense strand 32 P-RNA of fragment 1 (Fig. 1c), as in ref. 24. Autoradiography was for 1-5 days. Lane 1, KG1; 2, KC122; 3, EBV transformed B lymphoblasts; 4, EM3; 5, K562; 6, foreskin fibroblasts, passage 5; 7, COLO-320 neuroendocrine carcinoma; 8, 293 embryo kidney cells. Lanes 1, 2, 4 and 5 contain RNA from myeloid leukaemia cell lines. **C,** Map of the human *c-myc* gene. The three exons, the P1 and P2 promoters and fragments 1-3 which were used as hybridization probes, are marked.



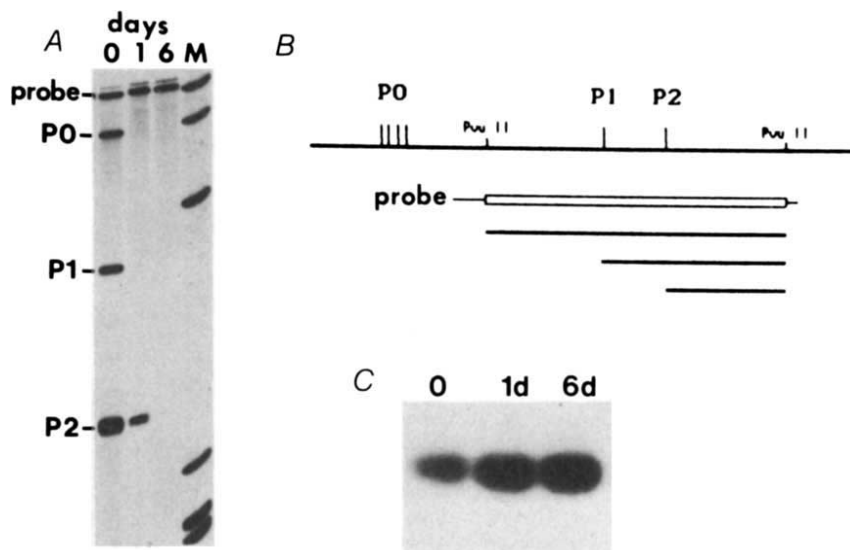
F9 embryonal carcinoma and mouse erythroleukaemia cells⁹. We have investigated transcription of the amplified *c-myc* genes in HL60 using a nuclear runoff assay. In this assay, short labelled RNAs (approximately 100 bases) are synthesized by polymerases *in situ* and hybridized to an excess of a cloned probe. Assuming a constant elongation rate, the hybridization signals from different probes reflect the number of RNA polymerase molecules present in corresponding regions of the gene. We initially examined transcription in four regions of the *c-myc* gene, labelled a-d in Fig. 1, using single-stranded M13 probes for both strands. The cloned restriction fragments were of similar

length so as to be approximately equally efficient hybridization targets.

As shown in Fig. 1, transcription of exons 1 and 2 is highly strand-specific, and the amount of exon 1 transcription is greater than that of exon 2 by an average of 3-fold (range 2-5) in uninduced cells and 15-fold (range 9-24) in induced cells. Each of these values is the average of four independent determinations made by densitometry of autoradiographs. Background signal was not subtracted, so the average value of 15 for the ratio of exon 1 to exon 2 transcription in differentiated cells is a minimum estimate. To ensure that the high exon 1 signal is not due to

Fig. 3 A, S_1 analysis of *c-myc* RNA during retinoic acid induced differentiation of HL60 cells. Markers (M) are 1,078; 872; 603; 310; 278 and 271 bases. B, Strategy for S_1 mapping. The probe is depicted with thin lines for M13 vector sequence and an open bar for *c-myc* sequence. The solid lines below represent S_1 resistant fragments characteristic of P0, P1 and P2 RNAs. C, Northern blot of actin RNA. Samples of 1 μ g of the same RNA samples as in Fig. 3A were analysed by hybridization to a 32 P-RNA probe from the chicken β -actin gene²⁶ (3 h exposure).

Methods. The probe in A was a uniformly labelled single-stranded fragment (prepared as in ref. 25) including 862 bases between two *Pvu*II sites and about 200 bases of M13 vector sequences. Samples of 1 μ g poly (A)⁺ RNA from uninduced cells labelled 0, and from cells treated with retinoic acid for 1 day (21 h) and 6 days were analysed. 0.5 ng of probe (25,000 c.p.m.) was hybridized to RNA in 10 μ l of 0.5 M NaCl, 20 mM PIPES, pH 6.4, 1 mM EDTA at 65 °C for 3 h, then digested in 100 μ l containing 350 mM NaCl, 30 mM Na-acetate, pH 4.5, 1 mM ZnSO₄, 1,200 U ml⁻¹ S_1 nuclease (BRL) for 40 min at 37 °C followed by ethanol precipitation and electrophoresis on a 4.5% acrylamide 7 M urea gel. (2.5 day exposure).



cross-hybridization with other transcribed sequences in the genome, we analysed runoff transcripts in another human cell line which was expected to have little or no exon 1 transcription. This line was the B-cell lymphoma Manca which has a single active *c-myc* allele that is disrupted in the first intron such that exons 2 and 3 are transcribed from cryptic promoters under the influence of the immunoglobulin heavy chain enhancer¹². In contrast to HL60, Manca, showed an excess of exon 2 transcription over exon 1 (Fig. 1). This result rules out the possibility of a highly transcribed sequence in the human genome which spuriously hybridizes to our exon 1 probe. In addition, 32 P-RNA corresponding to the anti-sense strand of probe C (Fig. 1) hybridizes to a unique band in Southern blots of HL60 and placental DNA (data not shown), thereby eliminating the possibility that the molar excess of exon 1 signal over exon 2 is due to the presence of amplified truncated *c-myc* genes containing exon 1 but not exon 2 sequences in HL60. The unequal signals obtained with exon 1 and exon 2 probes are not the result of differential hybridization efficiency since these two probes hybridized equally well to SP6 RNA from a cDNA clone (Fig. 1). The excess exon 1 transcription also does not represent artifactual initiation resulting from the nuclear isolation and runoff procedures, since the pattern of transcription in both uninduced (Fig. 1) and induced cells (data not shown) is unaffected by concentrations of sarkosyl (0.5%) which block Pol II initiation but not elongation¹³. The slight differences between the 'uninduced' and 'sarkosyl' runoff experiments shown in Fig. 1 are within the normal range of variation between experiments. Transcription of all parts of the gene assayed was sensitive to 2 μ g ml⁻¹ α -amanitin, demonstrating that these transcripts are synthesized by RNA polymerase II¹⁴ (Fig. 1). The high level of exon 1 transcription is apparently due to a high polymerase density throughout this exon and not a pile up of polymerases at its 5' end, since an equally high signal relative to probe length was obtained when the 5' 150 bases of probe C were deleted (data not shown).

Transcription of exon 2 sequences was down-regulated by over 90% when the cells were induced to differentiate for 2.5 days. This transcriptional regulation (Fig. 1) fully explains the reduction in steady state *c-myc* RNA in this cell preparation (see Fig. 1) without the need to invoke post-transcriptional con-

trol. Our result confirms a previous report²¹ in which a *v-myc* probe homologous to exons 2 and 3 was used to monitor transcription. As a positive control for the transcriptional activity of differentiated cells, we monitored the induction of a gene called Φ 11, which was selected from an HL60 cDNA library by a differential screening procedure (D.B., unpublished).

In contrast to exon 2, there was only a 10–20% down regulation of exon 1 transcription during HL60 differentiation in four experiments. At least part of the modest transcriptional down regulation of exon 1 is due to reduced transcription from upstream of the gene (see below). These results suggest that the major *c-myc* promoter, P2, is not turned off during HL60 differentiation. We cannot eliminate the possibility that the P1 promoter is turned off, since it seems to be a minor transcriptional start site based on steady state RNA measurements (see Fig. 3 below). The maintenance of a high transcription rate of exon 1 in differentiated cells while exon 2 transcription declines to background levels suggests that inhibition of elongation between exons 1 and 2 is an important component of the *c-myc* down-regulation in HL-60.

An unexpected result of the runoff assay was the detection of transcription upstream of exon 1 on both strands, at a level almost as great as that of exon 2. The observed hybridization is not due to homology with another part of the genome since nick-translated fragment *b* (Fig. 1) hybridized to a unique sequence in a Southern blot of genomic DNA (data not shown). In addition, the bidirectional transcription signal does not result from a lack of strand specificity of the probes, as demonstrated by asymmetric hybridization of SP6 RNA synthesized from a cDNA clone (Fig. 1). The origin of anti-sense transcripts was mapped to a *Sma*I-*Nae*I fragment that extends from 101 bases upstream of P1 to 49 bases downstream of P2 (data not shown). This fragment contains two TATA box homologies which might promote transcription: a GATAAA at 2,483–2,488 and a TATAAA at 2,298–2,303 of the sequence in ref. 22 as well as the distal element consensus, CCGCCC at 2,531–2,536. Divergent transcription from the area of the major promoter of the mouse dihydrofolate reductase (DHFR) gene has also been observed²³. Perhaps these two cases of divergent transcription are indicative of a more widespread lack of absolute polarity in promoter function. Anti-sense transcription originating about

100 bases upstream of the major mouse DHFR promoter produces a cytoplasmic, spliced polyadenylated RNA with an open reading frame. We have been unable to detect such a stable anti-sense RNA from the *c-myc* gene in HL60 or human fibroblasts; therefore, we presume that the anti-sense transcripts are very unstable.

In contrast, we did detect sense strand transcripts of the region upstream of P1 in steady-state RNA. Northern blots of HL60 RNA revealed a 3.1-kilobase (kb) *c-myc* RNA which hybridizes to a probe (Fragment 1, Fig. 2C) spanning 409–608 bases upstream of the P1 promoter (Fig. 2A, lane 1). A minor 2.5-kb RNA is also detected by this upstream probe. The 3.1-kb species also hybridized to exon 2 and exon 3 specific probes (Fig. 2A, lanes 2 and 3). Full length cDNAs of both these RNA species have been isolated and characterized, and the 5' ends of these RNAs were mapped to a promoter called P0 located 550–650 bases upstream of P1¹⁹. Several human cultured cell lines as well as primary fibroblasts were surveyed by Northern blot hybridization with the upstream probe, and all of them contain the 3.1-kb transcript (Fig. 2B), although usually as a lower proportion of *c-myc* RNA than observed in HL60. Thus, transcripts initiating upstream of the first exon appear to be found wherever the *c-myc* gene is expressed.

The regulation of P0, P1, and P2 RNAs during differentiation of HL60 into granulocytes was examined using an S1 protection assay (Fig. 3B). The results (Fig. 3A) show that steady-state levels of P0, P1 and P2 RNA are coordinately reduced when the cells differentiate. RNA levels declined to less than 10% of their original amounts after 21 hours when there is no overt morphological differentiation, and to undetectable levels after 6 days when differentiation is complete. As a control for the integrity of these RNA samples, a Northern blot was hybridized to an actin probe, and a slight increase in the amount of actin RNA was observed in the differentiated HL60 cells (Fig. 3C).

The relative amounts of RNA from the three *c-myc* promoters in HL60 were measured by cutting out the gel bands in Fig. 3A, lane 0 and counting them. After compensating for the number of labelled nucleotides in each fragment, it was calculated that P0, P1, and P2 contributed 7%, 12% and 81% of *c-myc* RNA respectively. The small amount of steady-state P0 RNA contrasts with the relatively high transcription rate from this promoter as judged by the nuclear runoff assay. This result implies that the majority of P0 transcripts are less stable than P1/P2 transcripts.

We next examined whether HL60 differentiation was accompanied by a change in the chromatin fine structure of the *c-myc* gene. DNase I hypersensitive sites in the *c-myc* gene of undifferentiated and differentiated HL60 cells were mapped using the indirect end-labelling strategy in Fig. 4A. The result demonstrates two major differences in chromatin structure on treatment with retinoic acid for 3 days. Hypersensitive site 2 (II₂ in ref. 28) which is located about 100 bases upstream of the P0 promoter disappears, as previously reported²⁹, whereas site 4 located in the first intron is enhanced relative to the other sites upon differentiation. Note also that the relative intensities of site 1 (1.85 kb upstream of P1) and site 3 (between P1 and P2) are unchanged after retinoic acid treatment. The disappearance of site 2 correlates with turn-off of the P0 promoter (Figs 1 and 3) which appears to be regulated independently of the major promoters P1 and P2. The persistence of site 3 (III₂ in ref. 28) correlates with maintenance of a high exon 1 transcription rate in differentiated cells (Fig. 1).

The position of hypersensitive site 4 about 300 bases into the first intron, and its induction during differentiation suggest that it may be associated with the block to elongation of transcription between exons 1 and 2. We therefore analysed transcription of the intron 1 sequences around this hypersensitive site in differentiated HL60 cells (Fig. 4b). The results show a sharp drop in the transcription downstream of probe A (Fig. 4b) which extends to the *Pvu*II site 40 bases upstream of the exon 1 splice donor. The apparent site of the transcriptional block

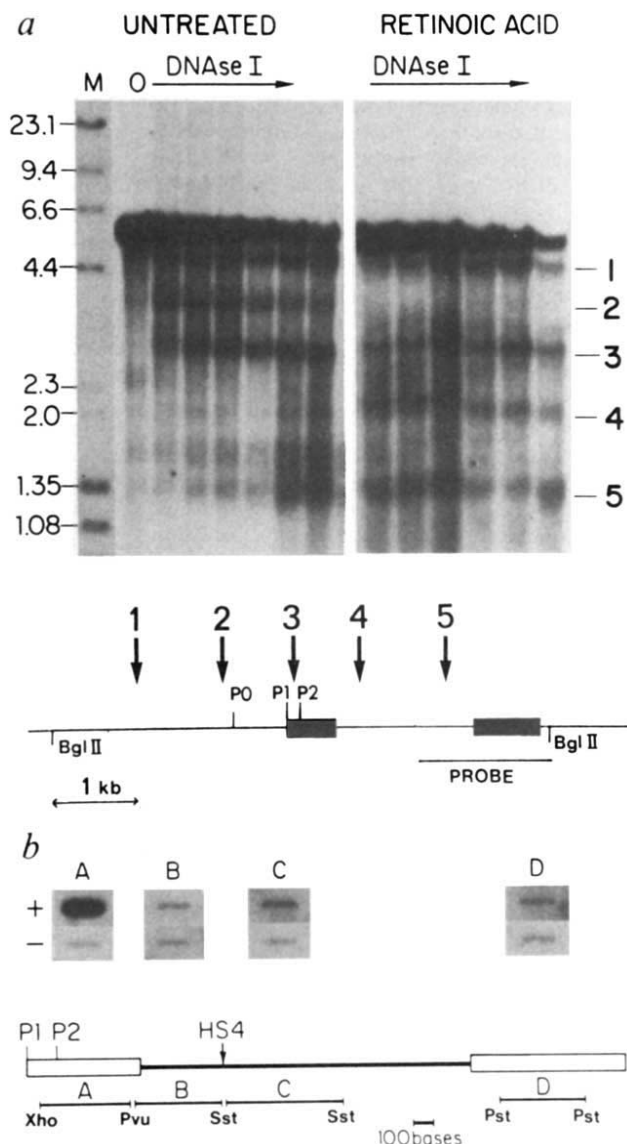


Fig. 4 *a*, DNase I hypersensitive sites in differentiating HL60. *b*, Run-off transcription of HL60 cells induced with retinoic acid for 2.5 days.

Methods. *a*, DNA from DNase I digested nuclei was restricted with *Bgl*II, blotted and hybridized²⁷ to the nick translated 1.5 kb *Sac*I fragment spanning exon 2. The map shows the 5' portion of the gene including the first two exons and marks the five hypersensitive sites. (The minor sites II₁ and III₁²⁸ were not clearly resolved.) Lane M, marker; Lane O, no DNase I. Arrows indicate increasing extents of DNase I digestion. HL60 was induced with 1 μ M retinoic acid for 3 days. Methods for *b* were as in Fig. 1. + and - refer to probes which detect sense and anti-sense transcripts, respectively.

Lengths of probes: A, 443; B, 430; C, 606; D, 414 bases.

therefore maps to the 3' end of exon 1 or the 5' end of intron 1 several hundred bases upstream of hypersensitive site 4. One testable hypothesis is that sequences near this hypersensitive site function at a distance to influence the process of termination which occurs upstream. It is of interest that several stretches of T residues occur in the region of the elongation block defined in Fig. 4b: T₇, T₄ and T₇ occur 17, 64 and 74 bases downstream of the *Pvu*II site near the end of exon 1. Such sequences have previously been implicated in the termination of transcription. Pol III terminates at the 3' end of *Xenopus* 5S RNA genes within clusters of four or more T residues³⁰. Furthermore, in two cases of termination by Pol II the phage λ 4S gene³¹ *in vitro* and the human gastrin gene *in vivo*³², the 3' termini map to clusters of

five or more T residues. Similarly, premature termination downstream of the adenovirus 2 major late promoter *in vivo* occurs near a cluster of 5 T residues^{33,34}. Further analysis is required to establish whether the T-rich sequence near the exon 1-intron 1 border of the *c-myc* gene also functions as a terminator.

Our nuclear runoff analysis of *c-myc* transcription in HL60 led to the discovery of a novel *c-myc* RNA initiating several hundred bases upstream of the major promoters. In addition, perhaps the most striking feature of the transcription pattern revealed here is the molar excess of exon 1 over exon 2 transcription observed in HL60 (Fig. 1) and also in HeLa and COLO 320 cells (data not shown). In this respect, our results differ from those of Blanchard *et al.*¹¹ who concluded that there was equimolar transcription throughout the *c-myc* gene in Chinese hamster lung fibroblasts. The explanation for this discrepancy could be the different cell types used. However, it is possible that the results of Blanchard *et al.* were biased against exon 1 by using non-homologous human probes. Exon 1 sequences have diverged considerably more between human and mouse than have the protein coding sequences of exons 2 and 3³⁵. The excess of exon 1 over exon 2 transcription which is accentuated from approximately 3- to 15-fold when HL60 cells differentiate, suggests a block to transcriptional elongation which can be modulated to control synthesis of full-length *c-myc* RNA. We have mapped the site of this block to the region of the boundary between exon 1 and intron 1. Although premature termination at this site seems likely, we cannot rule out the alternative of transcriptional pausing. In either case an elongation block, rather than decreased initiation, seems to account for the reduced exon 2 transcription in differentiated HL60 cells, and hence the reduced steady-state *c-myc* RNA level. Control of gene expression by blocking elongation is a flexible mechanism that allows modulation of transcription without requiring assembly or disassembly of the transcriptional apparatus. It will be of interest to determine whether this mechanism operates in other eukaryotic genes whose transcription is rapidly regulated.

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Idealization of the hydrophobic segment of the alkaline phosphatase signal peptide

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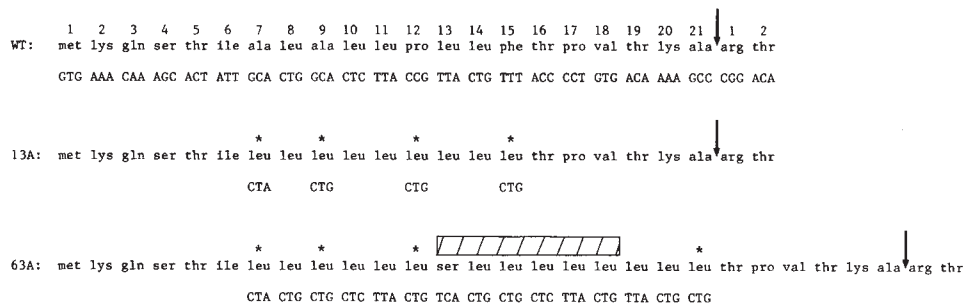
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Proteins secreted by prokaryotic cells are synthesized as precursors containing an amino-terminal extension sequence or signal peptide¹. Although these signal peptides share little primary sequence homology, recent studies suggest that they function via common pathways during the transport process² and that a common element may reside in their secondary structural characteristics³⁻⁷. We are investigating the role of an idealized hydrophobic sequence with high potential for α -helix formation in the *Escherichia coli* alkaline phosphatase signal peptide. Here, amino-acid substitutions were made using site-directed mutagenesis to produce a mutant signal sequence containing nine consecutive leucine residues in the hydrophobic core segment. Transport studies with this mutant precursor indicate that mature alkaline phosphatase is correctly targeted to the *E. coli* periplasm and that processing of the precursor to the mature form of the enzyme is extremely rapid. In contrast, processing is slowed when the mutant signal sequence is lengthened by the insertion of five additional leucine residues and one serine.

The alkaline phosphatase signal peptide, like other signal sequences, is characterized by a positively charged amino-terminus, a central hydrophobic core and a cleavage site recognized by the signal peptidase¹. Previous studies suggest that the hydrophobicity⁸ and helix-forming potential^{5,6,9} of the residues in the core region are important to signal peptide function. The unambiguous evaluation of the importance of these features has been complicated, however, as these studies often involve peptides of varied amino-acid composition. To optimize the potential to form an α -helical, hydrophobic domain and minimize problems in interpretation due to heterogeneity in amino-acid content, we have designed a mutant alkaline phosphatase signal sequence having a core region consisting of nine leucine residues. Leucine is an ideal amino acid for this approach as it is hydrophobic, has a substantial propensity to adopt an α -helical conformation^{10,11} and, fortuitously, already occurs five times in the alkaline phosphatase signal peptide. A similar approach has been used successfully to determine the requirements for amphiphilic secondary structures in peptide hormones, using chemically synthesized analogues¹².

The mutant signal peptide, denoted 13A (see Fig. 1), was constructed by making single amino-acid substitutions during each of four successive rounds of site-directed mutagenesis. In each round, a synthetic oligonucleotide primer containing the DNA sequence for the desired amino-acid substitution was annealed to single-stranded phage DNA from M13mp19 into which the structural gene¹³ for alkaline phosphatase (*phoA*) had been cloned. After elongation of the mutagenic primer and a second 'universal' primer¹⁴ located 5' to it, followed by ligation, the double-stranded phage DNA was used to transform *E. coli* strain AW1043F' Amp ($\Delta lac galU galK \Delta (leu-ara) phoA-E15 proC :: Tn5 F' Amp$). Mutant phage were identified by screen-

Fig. 1 Amino-acid and DNA sequences of wild-type (WT) and mutant alkaline phosphatase signal peptides. The amino-terminal 23 codons of the precursor DNA are shown in sense-strand polarity, together with the corresponding amino-acid residues and the signal processing site (arrow). Amino-acid substitutions used to construct mutants 13A and 63A are indicated by asterisks. Residues inserted into the hydrophobic core of the mutant 63A sequence are indicated by a hatched bar, and the DNA sequence repeated in this mutant is underlined. The DNA sequences of mutants 13A and 63A are identical to wild type except for the nucleotides shown.



ing plaque lifts with the ^{32}P -labelled oligomer used for mutagenesis, and subsequently verified by direct DNA sequencing¹⁵. Single-stranded template containing this mutant sequence was then used during the next round of mutagenesis.

During mutagenesis, an additional mutant containing an insertion of six amino-acid residues was isolated. This insertion resulted in a sequence of 14 leucine residues with a serine at the centre of this hydrophobic core (denoted 63A; see Fig. 1). The 63A mutant contains a 17-base-pair direct repeat, and probably arose from DNA replication or repair errors.

For transport studies, 2.5-kilobase DNA fragments containing the mutant *phoA* genes were subcloned into pBR322 between the *Bam*HI and *Hind*III sites and the resultant plasmids were used to transform *E. coli* strain AW1043 (Δ *lac galU galK* Δ (*leu-ara*) *phoA-E15 proC* :: Tn5). This strain contains a partially deleted alkaline phosphatase gene and a wild-type *phoR* regulatory gene^{13,16}.

Since a secretion-competent signal peptide directs export of alkaline phosphatase to the *E. coli* periplasm, we analysed the contents of this cellular compartment for the presence of the mature enzyme. Cells that harboured wild-type or mutant plasmids were labelled with ^{35}S -methionine for 1.5 min, washed, then treated with lysozyme to release the contents of the periplasm. Protein from the periplasmic fraction and an aliquot of whole cells were immunoprecipitated with rabbit anti-alkaline phosphatase antiserum, then analysed by SDS-polyacrylamide gel electrophoresis and autoradiography. Analysis of the periplasmic fraction for mutant 13A (Fig. 2) demonstrated that

mature alkaline phosphatase was transported efficiently and correctly and localized within the periplasmic space. The protein from this fraction co-migrated with purified bacterial alkaline phosphatase (data not shown). No accumulation of precursor was observed in the whole-cell sample from this mutant.

Analysis of mutant 63A (Fig. 2) also showed release of mature alkaline phosphatase into the periplasm but, in contrast to 13A, some accumulation of precursor in immunoprecipitates of whole cells was observed. We have found that the precursor form of the 63A mutant has a slightly greater electrophoretic mobility than the wild-type precursor; this may reflect changes in SDS binding caused by the introduction of extra hydrophobic residues rather than aberrant processing. Similar changes of electrophoretic mobility for mutant precursors have been noted in other systems¹⁷.

To assess the effect of the signal mutations on alkaline phosphatase processing, the rate of conversion of the precursor to the mature form was determined in pulse-chase experiments. Cells were radiolabelled with ^{35}S -methionine for 40 s, then chased with cold methionine for the times indicated in Fig. 3. The chase was stopped by adding trichloroacetic acid (TCA), then the cells were extracted with acetone. After extract solubilization, alkaline-phosphatase-immunoreactive material was isolated and analysed by SDS-PAGE and autoradiography. Analysis of the ratio of the precursor to the mature form of alkaline phosphatase in the pulse-chase experiment shown in Fig. 3 indicates that cleavage of the mutant 13A signal peptide was rapid. Within the detection limits of our experiment, there was

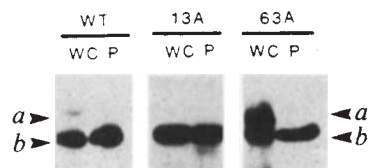
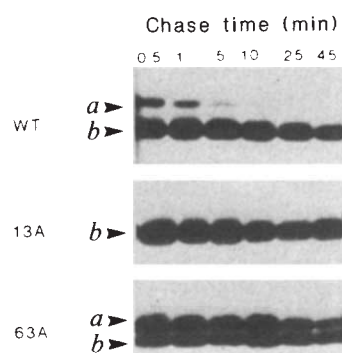


Fig. 2 Localization of alkaline phosphatase in *E. coli* transformed with wild-type, mutant 13A or mutant 63A plasmids. Arrows indicate the positions of the precursor (*a*) and mature forms (*b*) of alkaline phosphatase. WC, whole cell; P, periplasm.

Methods. Cultures of *E. coli* were washed and resuspended in MOPS (morpholinopropanesulphonate) medium²¹ supplemented with amino acids at $20 \mu\text{g ml}^{-1}$, minus methionine. The cells were labelled with $100 \mu\text{Ci } ^{35}\text{S}$ -methionine for 1.5 min. Labelling was stopped by adding ice-cold 200 mM sodium azide, 10 mM dinitrophenol and $200 \mu\text{g ml}^{-1}$ chloramphenicol as described previously². Half the culture was pelleted and washed with cold acetone. Protein was solubilized with 10 mM Tris pH 8.0, 1% SDS, 1 mM EDTA by boiling for 3 min. Following dilution by the addition of 50 mM Tris pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 2% Triton X-100, the clarified supernatant was immunoprecipitated essentially as described by Ito *et al.*²²; this constituted the whole-cell fraction. The remaining portion of the culture was washed with 33 mM Tris pH 7.5, 30 mM NaCl, then resuspended in 20% sucrose, 30 mM Tris pH 7.5, 1.0 mM EDTA containing $50 \mu\text{g ml}^{-1}$ lysozyme for 20 min on ice²³. After centrifugation, the supernatant which constituted the periplasmic fraction was immunoprecipitated²². The immune precipitates from whole cells and the periplasm were then analysed by SDS-PAGE under reducing conditions²⁴.

Fig. 3 Kinetics of alkaline phosphatase maturation in *E. coli* strains carrying the wild-type, mutant 13A or mutant 63A plasmids. Arrows indicate the positions of the precursor (a) and mature forms (b) of alkaline phosphatase.

Methods. Cultures were washed and resuspended in MOPS medium²¹ supplemented with amino acids at 20 $\mu\text{g ml}^{-1}$, minus methionine. The cells were labelled with 100 $\mu\text{Ci } ^{35}\text{S}$ -methionine for 40 s, and the chase was initiated by adding cold methionine to a final concentration of 4 mg ml^{-1} . At the times indicated, an aliquot of cells was precipitated in 5% TCA, then washed with cold acetone. Protein was solubilized, immunoprecipitated²² with anti-alkaline phosphatase antiserum, and analysed as described in Fig. 2 legend.



no accumulation of the 13A precursor. The accelerated rate of maturation of alkaline phosphatase encoded by the mutant 13A gene relative to that of alkaline phosphatase encoded by the corresponding wild-type gene may reflect optimization of an important recognition element or stabilization of an active conformation. In contrast, the kinetics of maturation of the 63A alkaline phosphatase gene product were slower than those for the wild-type gene and some mutant precursor was observed throughout the experiment. Inclusion of a serine in the hydrophobic core sequence and/or lengthening of this region thus seem to decrease the efficiency of processing and transport of the enzyme.

The above experiments were carried out using bacteria carrying a multi-copy plasmid bearing the wild-type or mutant alkaline phosphatase gene. Cellular transport factors may become limiting in a multi-copy situation where large amounts of a specific, secreted protein are being produced and the extent to which the cellular transport system is overburdened may contribute to the observed differences in processing rates. The wild-type, 13A and 63A plasmids are identical except in the regions encoding the signal peptide hydrophobic core. However, it is possible that, because of their location in the 5' end of the gene, these signal mutations could have nucleotide sequence-mediated effects on the degree of translation. The predicted minimum-energy structures¹⁸ for the 5' ends of the wild-type and mutant messenger RNAs were found to differ somewhat. It is possible, therefore, that translation initiation occurs at different rates in the mutants^{19,20}. Parallel experiments in single-copy systems will allow definitive examination of this issue.

The present results demonstrate that a signal peptide containing a poly-leucine core region efficiently supports export of alkaline phosphatase to the *E. coli* periplasm. The signal sequence of this mutant (13A) differs from the wild type by four amino-acid substitutions. The complete retention of biological activity despite multiple residue changes in the mutant enzyme underlines the importance of overall structural features as opposed to primary sequence. Furthermore, the interpretation

of the effects of individual substitutions can be misleading: for example, we have found that the individual substitution of phenylalanine for leucine (residue 15; see Fig. 1) in the alkaline phosphatase signal sequence retards precursor processing although export does occur (data not shown). The detrimental perturbation produced by this single mutation appears to be eliminated by the complete idealization of the region.

Extending the total length of the signal sequence by six residues may impair the orientation of the cleavage site, leading to the diminished rate of processing of the mutant 63A precursor. In addition, the inclusion of an extra serine, with its polar hydroxyl group, may decrease the effective length of the hydrophobic, potentially helical segment between this residue and the polar amino-terminus. Characterization of several export-defective mutants of maltose-binding protein indicates that the length of an uninterrupted hydrophobic domain is critical to function⁵.

In previous studies, correlations have been observed between the predicted secondary structure of signal peptides, transport activity, and the conformation of synthetic peptides corresponding to these sequences^{5,6,9}. We have now used a new approach to delineate the relationship between macromolecular structure and the function of signal peptides—that is, we have idealized the core segment to maximize the potential for formation of a hydrophobic α -helix. The results demonstrate that optimization of this structural unit is sufficient for transport and processing, and that specific residues are not required in the hydrophobic domain of the alkaline phosphatase signal peptide. This modelling approach can now be extended to other regions of the signal peptide and the mature protein.

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Stringent control of initiation of chromosomal replication in *Bacillus subtilis*

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Interconnecting regulatory networks are characteristic of probably all cellular systems. Such 'regulons' allow the cell to respond in a coordinated manner to changes in its environment, including a rapid return to balanced growth after stress^{1,2}. A regulon is usually composed of diverse metabolic pathways whose activities are amplified or inhibited by regulatory proteins, through the action of a small molecule, an alarmone³. Thus, for example, when the level of any aminoacyl transfer RNA becomes limiting in the bacterial cell, the stringent response is initiated; this is mediated through the nucleotide ppGpp and leads to the inhibition of synthesis of several major macromolecules, including stable RNA^{4,5}. Surprisingly, the key control point in the cell cycle, initiation of DNA replication, has not previously been implicated in the stringent response. Using a *Bacillus subtilis* mutant in which the transcriptional step of initiation can be studied independently of any requirement for protein synthesis⁶, we now show that initiation of chromosomal replication is indeed subject to stringent control.

The synthesis of a specific RNA-DNA co-polymer containing RNA and DNA sequences has been correlated with the transcriptional step required for initiation in *B. subtilis*^{7,8}. Moreover, a ribosomal RNA operon, *rrnO*, has been identified within the origin region of the chromosome⁹. These observations suggested to us that initiation of DNA replication, which may involve the synthesis of a stable RNA, was also likely to be subject to stringent control.

To induce the stringent response, we used *O*-methylthreonine (*O*-MeT), an inhibitor of isoleucyl tRNA synthetase, or an aminoacyl hydroxamate. These drugs promote the accumulation of large amounts of ppGpp and inhibit synthesis of stable RNA in a *relA*⁺ strain^{10,11}. In contrast, in a *relA*⁻ strain, the rate of RNA synthesis continues relatively undisturbed under these conditions. Figure 1 shows the results obtained with strains OMM242(*dna37*, *thy*⁻, *trp*⁻, *relA*⁺) and OMM243(*dna37*, *thy*⁻, *trp*⁻, *relA*⁻).

We tested the effect of the drugs on initiation of chromosomal replication and, for that purpose, replication rounds were synchronized in the temperature-sensitive *dna37* mutant of *B. subtilis*. As described previously¹², after return to the permissive temperature, DNA synthesis is synchronized for at least two rounds of replication. Under these conditions, initiation of the first round proceeds even in the absence of protein synthesis. However, the first round of DNA replication is blocked in the presence of inhibitors of transcription, for example, rifampicin and streptolydigin. Thus, we were able to test the effect of *O*-MeT on the transcriptional step quite independently of any requirement for protein synthesis. When added at the time of initiation, *O*-MeT, completely abolished DNA synthesis in the *relA*⁺ strain (Fig. 2a). To exclude the possibility that the drug was inhibiting elongation of DNA replication or the uptake of the labelled precursor, rather than inhibiting initiation, control experiments were done using exponentially growing cultures in which either the rate of incorporation of ³H-adenine into DNA or the amount of residual DNA synthesis in uniformly labelled cells was measured after the addition of *O*-MeT or chloramphenicol, which also blocks chromosomal initiation. In both cases, the effects of *O*-MeT and chloramphenicol were identical, indicating no major effect of *O*-MeT on uptake or DNA replica-

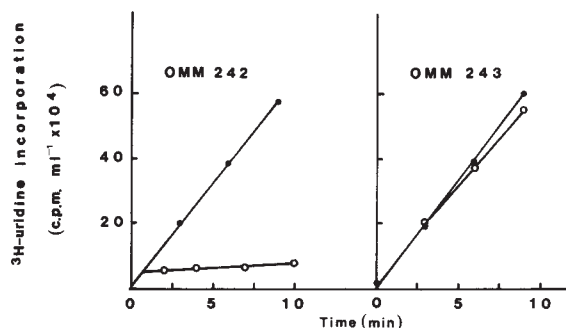


Fig. 1 Effect of *O*-MeT on RNA synthesis in *B. subtilis* OMM242 (*dna37* *thy*⁻ *trp*⁻ *relA*⁺) and OMM243 (*dna37* *thy*⁻ *trp*⁻ *relA*⁻). A derivative of OMM242 carrying the *relA*⁻ locus was constructed by transformation from strain BR17 (ref. 23). Strains OMM242 and OMM243 were grown at 30 °C in Spizizen medium²⁴ containing glucose (0.5%), casein hydrolysate (0.02%) and the required supplements. At *t*₀, cells were labelled with ³H-uridine (24 Ci mmol⁻¹; 10 µCi ml⁻¹) and *O*-MeT (1 mg ml⁻¹) (Sigma) was added to induce the stringent response. At intervals samples were removed and radioactivity in the RNA was determined after precipitation with trichloroacetic acid (TCA). ●, Without *O*-MeT; ○, plus *O*-MeT.

tion *per se*. Moreover, in synchronized cells, when *O*-MeT was added after initiation, substantial levels of DNA synthesis were detected (Fig. 2a).

The effect of *O*-MeT was then tested in a *relA*⁻ derivative of *dna37*. As shown in Fig. 2b, initiation of DNA synthesis was in this case largely resistant to the presence of *O*-MeT. Using another inhibitor of tRNA synthetase, arginine hydroxamate, which also induces the stringent response in OMM242, we found that initiation of DNA replication was again blocked in the *relA*⁺ strain but not in the *relA*⁻ derivative.

It was important to establish whether DNA synthesis occurring in the *relA*⁻ strain in the presence of such drugs involved initiation from the normal origin of the chromosome. Therefore, we compared the first fragments to be replicated after initiation of DNA synthesis in the untreated *relA*⁻ strain and in the same strain exposed to the drug. Figure 3 shows that the earliest fragments to be replicated were in fact identical in both cases. Moreover, these fragments corresponded exactly to the origin region of the chromosome (ref. 13 and A.L., G.H. and S.J.S., in preparation).

All these results indicate that initiation is subject to stringent control and therefore imply that high levels of ppGpp inhibit chromosomal initiation. One way to test that possibility is to increase the concentration of ppGpp without inducing amino-acid starvation; this can be achieved by adding a drug which inhibits the normal degradation of ppGpp. We used levallorphan¹⁴, which completely inhibited initiation of DNA replication in OMM242 without affecting DNA synthesis. This result is consistent with our hypothesis. However, as the full effects of levallorphan might be quite complex, further work is needed to verify this conclusion.

Here, we have presented evidence that chromosomal initiation is subject to stringent control in *B. subtilis*. Our results also indicate that ppGpp might be involved in this process. Future work will be concerned with identifying the precise step of the initiation process that is regulated by the stringent response.

In a previous study, Orr *et al.*¹⁵ examined the factors required for the expression of initiation potential that accumulated in non-synchronized cultures of the *Escherichia coli* *dnaA46* initiation mutant. Those workers observed that, although initiation proteins could be expressed during amino-acid starvation in a *relA*⁻ strain, this was not the case in a *relA*⁺ strain. These results indicated that normal initiation in *E. coli* might also be subject

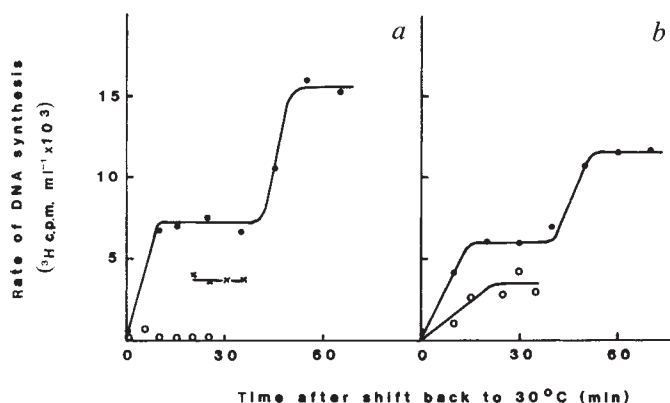


Fig. 2 Effect of *O*-MeT on initiation of DNA synthesis in cultures of *B. subtilis* OMM242 (a) and OMM243 (b) synchronized for DNA replication. An exponential culture of strain OMM242 and its derivative OMM243 ($A_{570}=0.175$) was shifted to 45°C for 30 min, diluted three times and shifted back to 30°C. Under these conditions, DNA replication is synchronized. At intervals, 2.5-ml samples were removed and ^3H -adenine (19 Ci mmol^{-1} ; $2 \mu\text{Ci ml}^{-1}$) was added. After 10 s, 2 min and 4 min, 0.5-ml samples were withdrawn and incorporation was stopped by adding 0.1 vol. of 1 mM NaN_3 . Samples were incubated at 80°C for 30 min in the presence of 0.4 M NaOH, neutralized by addition of 0.5 ml of 0.5 M HCl and precipitated with TCA. The rate of DNA synthesis is expressed as ^3H -adenine counts incorporated per ml of culture. ●, Control cultures; ○, plus *O*-MeT (1 mg ml^{-1}). The drug was added at 45°C 3 min before the return to 30°C. X, Addition of *O*-MeT (1 mg ml^{-1}) 15 min after the return to 30°C.

to stringent control, although the authors did not draw this conclusion. In the light of the results with *B. subtilis*, we re-investigated this possibility using an initiation mutant of *E. coli*, under similar conditions to those described in the present paper. Our preliminary results indicate that initiation of chromosomal replication in *E. coli* is also subject to stringent control. Interestingly, Hecker *et al.*¹⁶ have reported that the replication of the multi-copy plasmid pBR322 is also negatively controlled by the stringent response in *E. coli*.

In the present work, the effect of the *rel* system on initiation was observed under stress conditions mimicking amino-acid starvation. The question remains as to whether the stringent control system has any part in regulation of initiation under steady-state conditions. Bremer and colleagues¹⁷ have provided evidence that the levels of ppGpp are strictly correlated with ribosomal RNA synthesis in *E. coli* under steady-state growth conditions. Moreover, Van Verseveld *et al.*¹⁸ reported that ppGpp also negatively regulated cell division in *E. coli*, at least at extremely slow growth rates. It is therefore possible that the

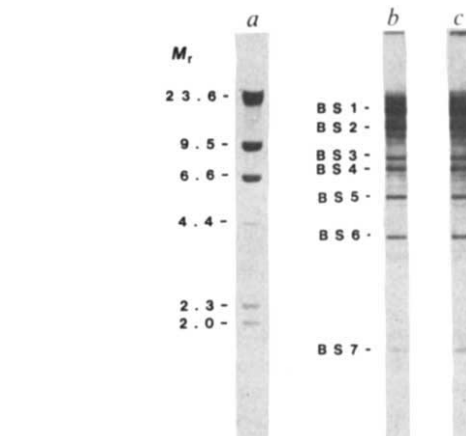


Fig. 3 Identification of the first DNA fragments to be replicated in *B. subtilis* OMM243 in the presence of arginine hydroxamate. A culture of strain OMM243 was shifted back from 45°C to 30°C as described in Fig. 2 legend, then pulse-labelled for 3 min with ^3H -thymidine (40 Ci mmol^{-1} ; $20 \mu\text{Ci ml}^{-1}$) in either the absence (b) or presence (c) of arginine hydroxamate ($50 \mu\text{g ml}^{-1}$; (sigma). The labelled DNA was purified and digested with *Bam*HI and *Sall*I. ^3H -labelled first replicating DNA fragments were separated by electrophoresis on a 0.8% agarose gel and detected by fluorography as described elsewhere²⁵. BS1-BS7 are fragments numbers from the replication origin of the chromosome. *Hind*III-digested λ fragments, indicated in a, are relative molecular mass (M_r) markers.

rel system, perhaps mediated through the effect of ppGpp, also has a role in the regulation of initiation during steady-state growth of bacteria.

As the initiation of chromosomal replication must be coordinated with the overall growth of a cell, it seems logical to suppose that this process also forms part of the stringent control system which includes the coordinated control of, for example, stable RNA, nucleotides, carbohydrates, fatty acids and peptidoglycan¹⁹. In eukaryotes there is evidence that the alarmone Ap4A, rather than ppGpp, is involved in some way in the regulation of DNA replication^{20,21} and in cellular proliferation²². It would be interesting to determine whether this particular phosphorylated nucleotide is also involved in the coordination of other macromolecular processes in a regulon analogous to the *rel* system, in higher organisms.

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